



**UNIVERSITE MONTPELLIER 2
SCIENCES ET TECHNIQUES DU LANGUEDOC**

THESE

pour obtenir le grade de

DOCTEUR DE L'UNIVERSITE MONTPELLIER 2

Formation Doctorale: Sciences des Aliments

Ecole Doctorale: Sciences des Procédés - Sciences des Aliments

Présentée par

Janchai POONLAPHDECHA

Le 8 December 2011

Biosynthesis of 2-acetyl-1-pyrroline, a potent flavour compound in rice : effect of salt stress during plant growth and some clues on the biosynthetic pathway

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M. Matthias WÜEST	Professeur, Université de Bonn, Allemagne	Examineur
M. Alain MORERE	Professeur, Ecole Nationale Supérieure de Chimie, Montpellier	Examineur
M. Alain AUDEBERT	Ingénieur de Recherche, CIRAD, Montpellier	Examineur
M. Renaud BOULANGER	Ingénieur de Recherche, CIRAD, Montpellier	Invité
M. Ziya GUNATA	Professeur, Université Montpellier 2	Directeur de thèse



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Janchai POONLAPHDECHA

Résumé Étendu



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Parmi plusieurs centaines de composés volatils de l'arôme du riz, il est aujourd'hui clairement établi que la 2-acétyl-1-pyrroline (2AP) est le composé aromatique clé. Il possède un seuil de perception très bas (0,1 ppb dans l'eau) et développe une note aromatique de type « pop-corn » dans le riz cuit. Il a été suggéré que la biosynthèse de cette molécule dans le riz est régie non seulement par des facteurs génétiques, mais également par des facteurs environnementaux, tels que les stress abiotiques par le sel et la sécheresse. Selon la littérature, le stress salin peut induire la synthèse de la 2AP en activant la synthèse de la proline, acide aminé majeur précurseur de la 2AP dans le riz. Cependant, les données sur l'effet d'un stress salin sur la relation entre la proline et la biosynthèse de la 2AP sont très peu nombreuses. De même, bien que des voies de biosynthèse de la 2AP dans le riz aient déjà été proposées (Bradbury *et al.*, 2008, Huang *et al.*, 2008) les étapes cruciales, à savoir la disponibilité de la 1-pyrroline et la source du groupe acétyle n'ont pas encore été démontrées. Cette thèse vise donc à répondre à ces questions.

L'effet du traitement salin pendant la croissance de plants de riz sur la synthèse de 2AP a été étudié sur deux modèles : des cultures en milieu liquide et des pots en serre. Les deux expérimentations ont été effectuées sur Aychade, une variété de riz aromatique provenant de la zone de Camargue en France.

Les plants de riz ont été cultivés en milieu liquide pendant une semaine à quatre concentrations de NaCl différentes: 1,7 ; 17 ; 85 et 170 mM. L'effet de la solution saline a été faible sur la croissance des jeunes plants de riz. La teneur en 2AP a été déterminée par une quantification précise nouvellement développée, dilution isotopique stable à travers extraction SPME et analyse CPG-SM/SM (Maraval *et al.*, 2010). Une corrélation positive entre la concentration en sel dans le milieu de culture et la teneur en 2AP des feuilles de riz a été observée. Le traitement salin augmente de manière significative les quantités en 2AP par rapport au témoin quelle que soit la concentration en sel appliquée dans le milieu de culture liquide. Inversement, la concentration en proline n'a augmenté qu'en présence des deux plus fortes teneurs en NaCl, 85 et 170 mM. Par conséquent, il est donc possible que la proline ne soit pas le facteur limitant pour la biosynthèse de 2AP dans des conditions de traitement salin modérées (de 1,7 à 17 mM de NaCl). Néanmoins, la biosynthèse de la 2AP est augmentée en présence de fortes teneurs en NaCl (de 85 à 170 mM NaCl) où une augmentation significative en concentration en proline a été observée. De plus, une diminution de la concentration en

acide γ -aminobutyrique (GABA) à 170 mM a été également pu être observée. Dans cette condition, la conversion du γ -aminobutyraldéhyde (GABald) en GABA par la bétaine aldéhyde déshydrogénase (BADH) peut avoir été limitée (Bradbury *et al.*, 2008), puisqu'une augmentation assez importante de la biosynthèse de 2AP a tout de même été observée.

Aychade a également été cultivée dans des pots, en sol, dans une serre à une seule concentration en NaCl, 30 mM. Selon la littérature cette concentration en sel, correspondant à une conductivité électrique (CE) de $3800 \pm 400 \mu\text{S}.\text{cm}^{-1}$, suffit à induire un stress salin. Les plantes, placées dans des bacs, ont été soumises à quatre traitements différents (T1-T4) selon le stade de croissance (phase végétative, reproductive et maturation) et la durée d'application (14 à 28 jours) de la solution saline. L'eau osmosée inversée ($\text{CE}_{\text{eau}} < 500 \mu\text{S}.\text{cm}^{-1}$) a été utilisée comme témoin (T0). En général, l'application de la solution saline pendant la culture des plants de riz n'a pas eu d'effet significatif sur la croissance des plantes et des composantes du rendement, sauf sur le poids mille grains (TGW) où une diminution significative a été observée lorsque le traitement salin avait lieu à la phase de reproduction. La date et la durée d'application de la solution saline pendant la croissance des plants de riz a entraîné des changements de teneurs en 2AP à la fois dans les feuilles et les grains. La plus forte concentration en 2AP a été observée dans les feuilles (de 5717 à 6168 $\mu\text{g}.\text{kg}^{-1}$ MS) provenant d'un traitement salin pendant la phase végétative (I) (du début tallage (DT) à la mi-tallage (MT)), c'est-à-dire pour le traitement T1, et pendant toute la phase végétative (du DT à l'initiation paniculaire (IP), traitement T3. Il est important de noter, qu'une augmentation significative en 2AP par rapport au témoin a été observée pour les grains provenant de tous les traitements salins. En particulier, l'application du sel pendant les phases végétative et reproductive a donné la plus forte concentration en 2AP dans les grains (998 et 859 $\mu\text{g}.\text{kg}^{-1}$ MS, respectivement). Le stress salin pendant la phase végétative (du DT à l'IP) a pu augmenter de façon significative la concentration en 2AP dans les feuilles et les grains. Par contre le traitement salin pendant la phase reproductive (de l'IP à FLO) a augmenté de façon significative la concentration 2AP seulement dans les grains. La quantité en proline a augmenté de façon significative seulement quand le traitement salin est appliqué durant la phase végétative. De même, la teneur en 2AP a augmenté dans ces plantes. Ces données corroborent des résultats précédemment observés dans les cals de riz où la proline a participé à la biosynthèse de 2AP (Yoshiashi *et al.*, 2002b). De façon surprenante il n'y a aucune relation entre la quantité de proline et celle de 2AP dans les grains bien qu'une augmentation significative de la teneur en 2AP dans les feuilles a été observée lors du traitement salin

pendant les phases végétative et reproductive. Deux possibilités peuvent expliquer ce phénomène. Soit la 2AP synthétisée dans les feuilles pourrait avoir été transportée dans les grains ou la proline a été transportée à partir des feuilles vers les grains où la synthèse de 2AP a eu lieu. Par ailleurs il a été remarqué que les changements en GABA, un des métabolites dans la synthèse de 2AP en relation avec l'activité de la *BADH2* (Bradbury *et al.*, 2008), ne peuvent pas être liée à la synthèse de la 2AP.

En conclusion de cette partie, nos résultats donnent quelques indications sur la conduite des conditions de la salinité pendant la culture du riz afin de stimuler la biosynthèse de la 2AP. La salinité pendant la phase végétative pourrait en effet favoriser la synthèse de ce composé dans les grains, sans avoir diminué le rendement en grains.

Dans la seconde partie de ce travail, l'origine du groupe acétyle dans la 2AP et le rôle de la 1-pyrroline sur la biosynthèse de cette molécule ont été étudiés dans des cals de riz produits à partir de la variété Aychade. Tout d'abord nous avons confirmé que la L-proline est capable d'augmenter la teneur en 2AP dans les cals de riz, en accord avec une précédente étude réalisée sur une autre variété de riz aromatique (Yoshihashi *et al.*, 2002b). Lorsque les cals de riz ont été additionnés de la 1-pyrroline, la concentration en 2AP a augmenté considérablement. Cette augmentation, dépendante du pH, a été plus forte à pH 8,0 qu'à pH 5,8. Une fraction plus importante de 1-pyrroline aurait pu être transformée en sa forme cyclique ouverte à pH 5,8 ce qui expliquerait la diminution en la teneur en 2AP. Le traitement thermique des cals de riz avant l'addition de 1-pyrroline a entraîné une diminution importante de la biosynthèse de 2AP suggérant que l'acétylation de la 1-pyrroline pourrait être d'origine enzymatique. Par ailleurs une relation dose-dépendante a été mise en évidence entre la teneur en 1-pyrroline et celle en 2AP. Les données ont tendance à montrer que le donneur acétyle n'est pas un facteur limitant pour la biosynthèse de la 2AP dans le riz. Cette hypothèse a été corroborée par les résultats de l'expérimentation avec les cals d'une variété de riz non aromatique Gladio x Fidji-K2. Lorsque les cals de riz de cette variété ont été supplémentés par de la 1-pyrroline (1000 ppm), la teneur en 2AP a été multipliée par 290. La teneur en 2AP était de $580 \mu\text{g.kg}^{-1}$, proche de celle observée dans les cals de riz de la variété Aychade, additionnés de 1-pyrroline. L'ensemble des résultats montrent que la 1-pyrroline est un facteur limitant pour la synthèse de 2AP dans le riz.

En ce qui concerne la source acétyle de la molécule de 2AP, les expérimentations ont été menées sur des cultures de cals de la variété Aychade. La supplémentation des cals de riz avec de l'acide pyruvique a entraîné une hausse sensible de la teneur en 2AP. L'acide pyruvique pourrait avoir été incorporé au groupe acétyle à travers d'intermédiaire pyruvaldéhyde.

La source du groupe acétyle a également été étudiée en supplémentant le milieu de culture des cals de riz par les composés marqués, [U- ^{13}C]6-glucose, [^{13}C]2-acétate de sodium et [^{13}C]4-octanoate de sodium. Dans l'expérimentation avec le [U- ^{13}C]6-glucose, 7 isotopomères possibles de [^{13}C]-2AP (m/z 111 à 117) avec plusieurs fragments d'ions (m/z de 68 à 73; m/z de 83 à 88) ont été observés. Cela pourrait s'expliquer par le fait que les atomes ^{13}C , ont été non seulement intégrés sur le groupe acétyle de la 2AP mais aussi sur le cycle de la 1-pyrroline à différentes positions. La voie de la glycolyse pourrait avoir générée des intermédiaires différents qui ont été utilisés dans la synthèse de la 2AP.

La détection de l'isotopomère [^{13}C] 2-2AP (m/z 113) et de l'ion m/z 45 montre que deux carbones marqués ^{13}C à partir du glucose ont été incorporés au niveau du groupe acétyle. Les expérimentations avec le [^{13}C] 2-acétate de sodium et le [^{13}C] 4 octanoate de sodium ont conduit à un isotopomère de m/z 113 par IE / SM toutefois de faible intensité. Une augmentation de 2 unités de masse des ions fils (m/z 72, 86) par rapport au 2AP non marqué (m/z 70, 84) a également été observée en IC-SM-SM. Cela tend à suggérer l'incorporation de deux carbones marqués en ^{13}C , dans le cycle de la 1-pyrroline sous forme acétyle au travers de l'acétyl CoA généré par la β -oxydation des acides gras.

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List of Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
2AP	2-acetyl-1-pyrroline
2AP-d2	2-acetyl-1-d2-pyrroline
ANOVA	Analysis of variance
BADH2	Betaine Aldehyde DehydroGenase
BT	Beginning Tillering
CI	Chemical Ionization
CID	Collision Induced Dissociation
CIRAD	Centre de Coopération Internationale en Recherche Agronomique pour le Développement
CMC	Carboxymethylcysteine
dabsyl	4-Dimethylamino) Azobenzene-4-Sulfonyl Chloride
DAC	Days After Culture
DAS	Days After Sowing
DVB/CAR/PDMS	divinylbenzene/ Carboxen/polydimethylsiloxane
DW	Dry Weight
EC	Electrical Conductivity
ECs	Electrical Conductivity of Soil
ECw	Electrical Conductivity of Water
EDTA	Ethylenediaminetetraacetic acid
EI	Electron Impact
ESP	Exchangeable Sodium Percentage
FAO	Food and Agriculture Organization
FAPRI	Food and Agricultural Policy Research Institute
fgr	fragrance gene
FID	Flame Ionization Detector
FLO	Flowering
FW	Fresh Weight
GABA	Gamma Aminobutyric Acid
GABald	γ -Aminobutyraldehyde
GC	Gas Chromatography
HPLC	High Liquid Chromatography Performance
HS	Headspace Solid
IBMM	Institut des Biomolécules Max Mousseron
LOD	Limits of Detection
LOQ	Limits of Quantification
LS medium	Linsmaier and Skoog medium
LSE	Liquid Solid Extraction
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MS medium	Murashige and Skoog medium
MT	Middle Tillering
NaOCl	sodium hypochlorite
NPD	Nitrogen Phosphorus Detector
OAV	Odour Activity Values

OECD	Organisation for Economic Co-operation and Development
P5C	Δ^1 -Pyrroline-5-Carboxylate
P5CDH	Pyrroline-5-Carboxylate Dehydrogenase
P5CR	Δ^1 -Pyrroline-5-Carboxylate Reductase
P5CS	Δ^1 -Pyrroline-5-Carboxylate Synthetase
PCA	Principal Component Analysis
PCI	Positive Chemical Ionization
PI	Panicle initiation
PTFE	Polytetrafluoroethylene
RO	Reverse Osmotic Water
RWC	Relative Leaf Water Content
SAFE	Solvent Assisted Flavor Evaporation
SDE	Steam Distillation/ Solvent Extraction
SHS	Static Headspace
SIDA	Stable Isotope Dilution Assay
SPE	Solid Phase Extraction
SPME	Solid Phase Microextraction
TGW	Thousand Grain Weight
TMP	Trimethylpyridine
UNCTAD	The United Nations Conference on Trade and Development

General Introduction

Fragrant rice varieties are widespread in Southeast Asia and have also gained wider acceptance in Europe and the U.S. (Hori *et al.*, 1992; 1994). Currently, the demand for fragrant rice varieties is increasing in both domestic and international markets. They determine the premium price in global trade. Nowadays consumers have become more conscious about the quality of the rice they consume. They prefer fragrant rice due to their characteristic and pleasant odor.

Among several volatiles from rice, 2-acetyl-1-pyrroline (2AP) imparts characteristic flavor of fragrant rice that distinguishes it from non fragrant cultivars (Buttery *et al.*, 1982; Grim *et al.*, 2001; Maraval *et al.*, 2008). Although genetic factors play a main role in defining rice aroma (Lorieux *et al.*, 1996; Bradbury *et al.*, 2008; Fitzgerald *et al.*, 2008a), however environmental factors and cultivation practices have been demonstrated to greatly affect the aromatic quality of rice (Champagne, 2008). The environmental factors such as climate, temperature, water supply and salinity of soil (Efferson, 1985; Ishitani and Fushimi, 1994) have often an impact on flavor of rice. In the field, Yoshihashi *et al.*, (2004a) reported that drought stress applied during the milky stage of the ripening phase led to an increase in 2AP content in the grains at harvest. Recent studies have reported that the soil salinity had a positive impact on the level of 2AP in the grains (Gay *et al.*, 2010).

Many studies reported that fragrant rice cultivars are quite salt-stress sensitive during the early seedling and reproductive stages (Heenan *et al.*, 1988; Lutts *et al.*, 1995). This results in a reduction in crop productivity when rice plant was stressed during those periods (Zeng and Shannon, 2001). One major challenge is to create varieties with high aromatic quality and good yield.

With regard to 2AP biosynthesis in rice it was demonstrated that proline was the major amino acid precursor (Suprasanna *et al.*, 1998; Yoshihashi *et al.*, 2002b). Response to water deficits and salinity stresses promote its accumulation together with γ -aminobutyric acid (GABA), a metabolite of proline (Delauney and Verma, 1993; Taylor, 1996; Kinnersley and Turano, 2000). Only few studies reported the effect of salt stress under controlled conditions on the biosynthesis of 2AP in relation with proline and GABA biosynthesis. Moreover although biosynthetic pathway of 2AP in rice has been proposed (Bradbury *et al.*, 2008; Huang *et al.*, 2008), almost nothing is known about last key step, i.e., availability of 1-pyrroline and source of acetyl group. These aspects have been studied during this work.

First, literature reviews of database of rice, salt stress and flavor of rice were presented.

In part 1, effect of salinity stress on 2AP, proline and GABA synthesis was studied in rice seedling and greenhouse conditions. Aychade, a fragrant rice variety from Camargue was used in the experiments.

In part 2, the influence of 1-pyrroline and origin of acetyl group were investigated. For this propose, rice callus were generated and supplemented with 1-pyrroline to understand its role in 2AP biosynthesis both in fragrant and non fragrant varieties. Origin of acetyl group of 2AP was mainly studied by generating rice callus with C13 labeled glucose and fatty acids.

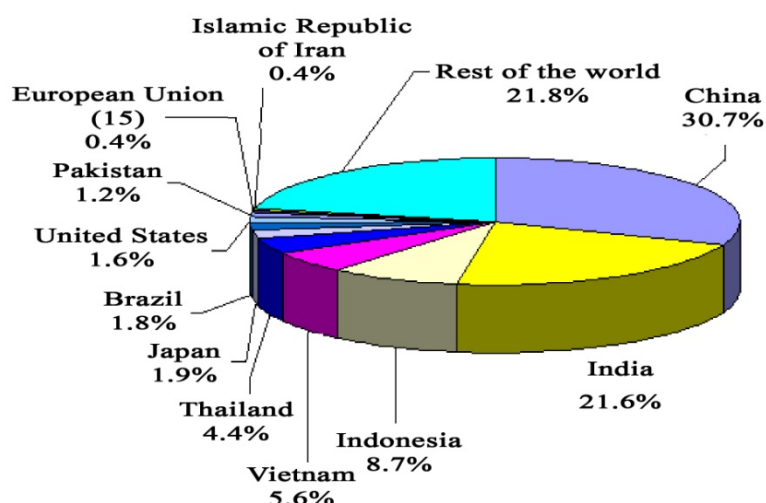
In the general discussion and conclusion, synthesis of entire works is presented and perspectives are also proposed.

Literature Reviews

1. Rice

1.1. Rice production and consumption in the world

Rice is a major food staple for over half of the world's population (Wu *et al.*, 2004). Rice is also the second largest produced cereal in the world, following wheat. During the beginning of the 1990s, annual production was around 350 million tons and by the end of the century it had reached 410 million tons. In 2010, world rice production was expected to increase rice production reaching 474 million tons (FAO, 2010). It is estimated, more than 148 million hectares area is under rice cultivation in 114 countries throughout the world (FAO, 2006). Production is geographically concentrated in Western and Eastern Asia harvesting more than 90% of the world's production of rice (**Figure 1**). The majority of all rice produced comes from India, China, Japan, Indonesia, Thailand, Burma and Bangladesh. China and India, which account for more than one-third of the global population (52.3 % over the 1999 - 2003 period) supply over half of the world's rice. Brazil is the most important non-Asian producer, followed by the United States, while Italy ranks first in Europe. Globally, rice provides 27% of dietary energy supply and 20% of dietary protein intake (Kueneman, 2006). For most rice-producing countries where annual production exceeds one million ton, rice is the staple food. Over 75% of the world's supply is consumed by people in Asian countries. In Bangladesh, Cambodia, Indonesia, Lao PDR, Myanmar, Thailand, and Vietnam, rice provides 50-80% of the total calories consumed (Pingali *et al.*, 1998). Notable exceptions are Egypt, Nigeria and Pakistan, where rice contributes only 5-10% of the per capita daily caloric intake. Approximately 405 million tonnes were consumed in 2001/02. China, India, Indonesia, Bangladesh and Vietnam are the largest consumers of rice. Global consumption of rice is forecasted to increase in the coming years, to 443 million tons in 2010 and to almost 478 million tons in 2018 (FAPRI, 2009), mainly as a result of a growing population.



Source: UNCTAD Secretariat from the Food and Agriculture Organization of the United Nations (FAO) data

Figure 1. Distribution of the world paddy rice production (average 1999-2003)

1.2. History and diversity of rice

It is believed that rice cultivation began simultaneously in many countries over 6500 years ago and its origins are from the tropical and subtropical Southeast Asia and Africa. The first crops were observed in China (Hemu Du region) around 5000 B.C. From there, derived species of Japonica and Indica expanded to Asian countries. The Asian rice (*Oryza sativa*) was adapted to farming in the Middle East and Mediterranean Europe around 800 B.C. Between 1500 and 800 B.C., the African species (*Oryza glaberrima*) propagated from its original center, the Delta of Niger River, and extended to Senegal. However, it never developed far from its original region (<http://www.unctad.org/>).

Rice belongs to the genus *Oryza* and the tribe Oryzeae of the family Gramineae (Poaceae) (**Table 1**). Today, most countries cultivate varieties belonging to the *Oryza* type, which has around twenty different species. Only two of them, *O. sativa* and *O. glaberrima* offer agricultural interest

1.2.1. *Oryza sativa*: common Asian rice, found in most producing countries, originated in the Far East at the foot of the Himalayas. Classification of rice cultivar (*Oryza sativa*) can be divided into three ecological varieties (OECD, 1999).

- *O. sativa japonica* grown on the China side of the mountains. Japonica is irrigated rice best grown in temperate zones and rainfed lowland rice of warm tropical zones with medium or short grains, are also called round grains.
- *O. sativa indica* grew on the India side. Indica is irrigated rice of grown in warm tropical and sub-tropical zones, with long, thin and flat grains. The indica type occupies ca. 80 % of the cultivated rice area in the world (Wu *et al.*, 1990).
- *O. sativa javanica* is cultivated only in Indonesia.

1.2.2. *Oryza glaberrima*: common African rice, originating from West Africa, covers a large region extending from the central Delta of the Niger River to Senegal. However, it never developed far from its original region.

Table 1. Scientific classification of rice (Datta, 1981)

Family	Gramineae or Poaceae
Tribe	Oryzeae
Genus	Oryza
Species	<i>O. sativa</i>
	<i>O. glaberrima</i>
Subspecies of <i>O. sativa</i>	<i>Oryza sativa</i> L. ssp. <i>japonica</i>
	<i>Oryza sativa</i> L. ssp. <i>indica</i>
	<i>Oryza sativa</i> L. ssp. <i>javanica</i>

O. sativa indica and *O. sativa japonica* are the most widely cultivated species. It is grown worldwide, particularly in Asian, North and South American, European Union, Middle Eastern and African countries. *O. Glaberrima*, however, is grown solely in West African countries. Nowadays, the Asian species (*O. sativa*) is cultivated far more than the African species (*O. glaberrima*), because of their higher yield and pleasant taste.

1.3. Morphology and development of the rice plant

Rice is a member of monocot and an annual plant. Rice is the only Graminea possessing both ligules and auricles, which allows its distinction from weeds at the seedling stage (**Figure 2c**) www.knowledgebank.irri.org. The components of the rice plant may be divided as follows.

1.3.1. Rice plant

Rice plants have tall stems hovering from one to five metres tall in deep water with each plant having a primary round and hollow stem. The actual plant itself has several tillers, long flat leaves with a terminal flowering head, or panicle with spikelets (**Figure 2**). The plant consists of vegetative organs (**Figure 2** and **2a**) such as roots, stems, leaves, and reproductive organs (**Figure 2b**). The reproductive organs are the panicle that makes up the spikelets that generate green and yellow flowers. The flowers form the grains of rice. Each panicle has 50 to 300 flowers (floret or spikelet) and is measured between 20 and 30 centimeters in length. A variety of differences in length, shape and angle of the panicles had been observed (<http://www.unctad.org>).

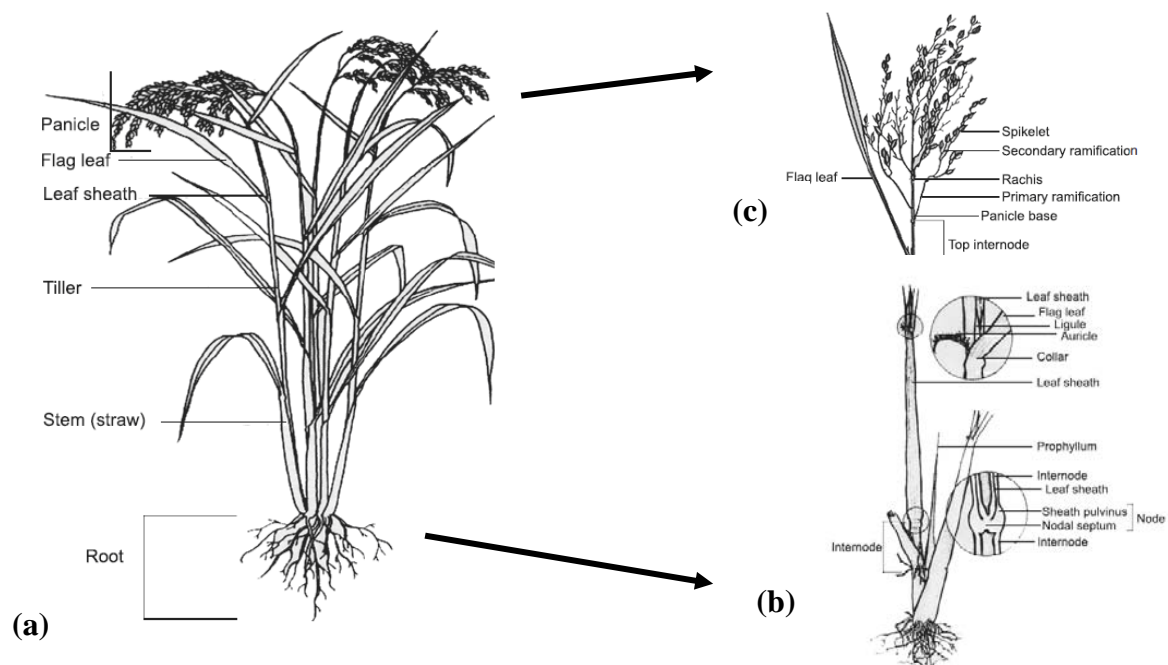


Figure 2. The components of the rice plant (a) vegetative organs (b) and reproductive organs (c). (Datta, 1981)

1.3.2. Rice grain

The grain is the seed of the rice plant, a fertilized and ripened ovule containing a live embryo capable of germinating to produce a new plant. It is composed of three main parts (**Figure 3**):

- **The rice envelope** made of glumes (large portions above the pedicels that link the spikelets to the secondary ramifications) and the two husks called palea and lemma. The awns are the prolongation of the ventral vein of the lower husk. The husks, which wrap the rice grain (caryopsis), will constitute the chaff when husking.
- **The endosperm** (the edible portion), contains starch, proteins, sugar, fats, crude fiber and inorganic matter. It will provide nutriment to the germinating embryo.
- **The embryo** is situated in the ventral part of the spikelet. The embryo contains the plumule (embryonic leaves) and the radicle (embryonic primary root).

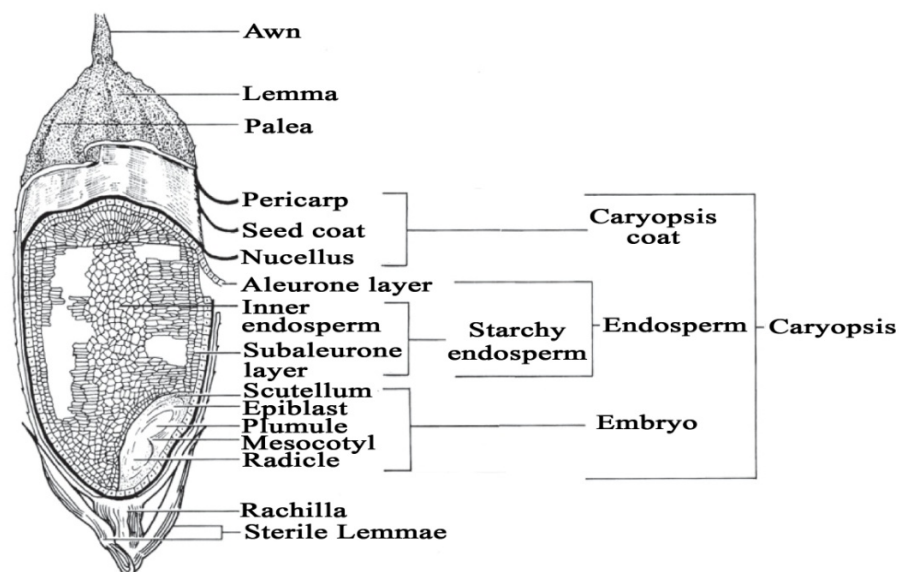


Figure 3. Structure of the rice grain (Datta, 1981)

1.4. The cycle of growth and development of the plant

The growing season of some traditional varieties is about 260 days, but is between 90 to 110 days for most modern varieties. The short growing season is a main factor that elevates cropping intensity (crops/ha/yr) (Dobermann and Fairhurst, 2002). The growth of the rice plant is divided into three phases as follows: vegetative, reproductive and ripening phase (**Figure 4**).

➤ **The vegetative phase** starts at seed germination and ends at the beginning of panicle initiation, during the late vegetative phase. The late vegetative phase starts at the tillering stage. Stem elongation begins late in the tillering stage and ends just before panicle initiation, which also signals the end of the vegetative phase. Period of this stage is generally 35 - 65 days.

➤ **The reproductive phase** begins at the panicle initiation and ends at flowering, usually taking 35 days. During this phase, the plant is not affected by photoperiod but is very susceptible to low temperatures, drought and salinity that can lead to the sterility of the reproductive organs, which means the grains will be empty.

➤ **The ripening phase** starts at flowering and ends at maturity. This stage usually takes 30 days; the rice seeds will eventually ripen, turning golden brown when fully ripe. Rainy days or low temperatures may lengthen the ripening phase, while sunny and warm days may shorten it.

The differences in growth duration are determined by changes in the length of the vegetative phase. The number of days in this phase varies according to variety, but it is also influenced by temperatures and the photoperiod (day - length), when the variety is susceptible, lengthens the phase. For example, low temperature or long day length can increase the duration of the vegetative phase. The relative length of the vegetative phase will define whether the variety has a short, medium or long growing cycle. While the number of days in the reproductive phase and the ripening phase are the same among most rice varieties (<http://www.knowledgebank.irri.org/>).

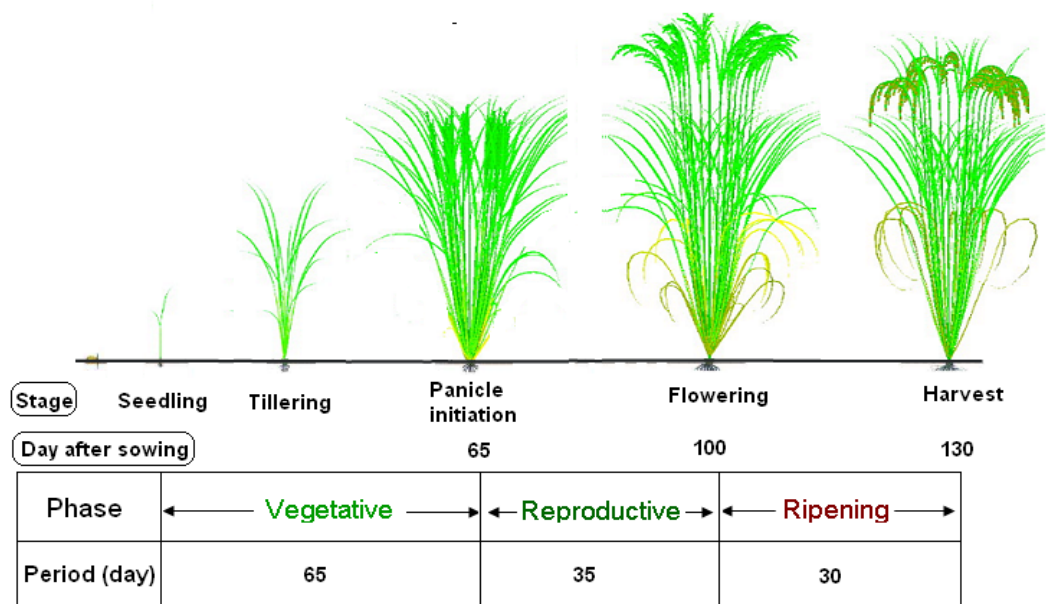


Figure 4. The life cycle of rice plant

1.5. Rice ecosystems

Rice is usually grown in tropical, semi tropical and temperate regions; mostly in coastal plains, tidal deltas, and river basins. The land needs to be submerged under water, thus it is necessary to have access to a supply of freshwater. Rice production systems are classified according to the water regime as a basis for the classification system, such as upland, irrigated, rainfed lowland, deep water (Datta, 1981). Additionally, the ecology of the mangrove swamp system attributes to deep water and salinity conditions that were also stated (Ferrero and Nguyen, 2004).

1.5.1. Upland rice ecosystem

Upland rice is often grown in hilly areas and grown without surface water, relying solely on the rainfall. An assured rainfall over a three to four month period is necessary as the crop is rainfed and the water supply is not controlled.

1.5.2. Irrigated rice ecosystem

Rice is typically grown with good water control and flooded throughout the growing season. The irrigated rice ecosystems are characterized by high cropping densities, with intensive use of agrochemicals, energy and water.

1.5.3. Rainfed lowland rice ecosystem

Rice is grown in standing water in fields with levees to impound natural rainfall. If rainfall is heavy, water may reach depths of 50 to 100 cm.

1.5.4. Deep water rice ecosystem

Rice is grown in river areas, with no structured water control and water submergence in depth exceeding 100 cm, for more than ten days and up to five months.

Based on the water management patterns for rice cultivation in the mid - 1990s, irrigated rice systems predominate, covering about 45 percent of the global rice area and generating around three quarters of the global output (**Figure 5**). Rainfed lowland rice production systems rank second in importance, covering around 30 percent of rice land base; followed by 10 percent of the upland rice ecology; 11 percent deep water ecosystems; and flooding ecology, with 4 percent of the world area under rice.

1.5.5. Mangrove swamp rice ecosystem

An ecosystem located in tidal estuaries, close to the ocean, consists of mangroves or former mangrove swamps. Successful cultivation of rice in the mangrove swamps depends on the length of the salt free period, which is the result of the exchange of the volume of fresh water available and salt water intrusion from the sea. Mangrove swamp soils are generally more fertile than those of other rice growing ecology because they collect regular silt deposits during annual flooding. However, salinity and acid sulphate conditions are the major problems encountered on adverse soils in mangrove swamp environments (<http://www.fao.org/>).

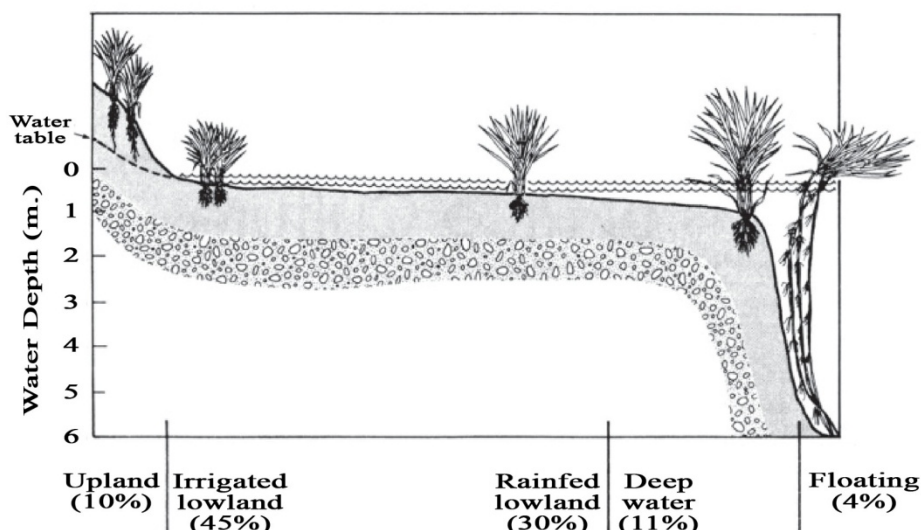


Figure 5. Rice production system classified by water management system (Datta, 1981).

1.6. Rice transformation and compositions

Paddy rice can be separated into hull and brown rice by hulling. Hull weight averages about 20% of the total grain weight. The brown rice can be further milled and polished to produce white rice (**Figure 6**).

Rice comprises approximately 80 - 85 % starch, 6 - 9% protein and 10 - 14% moisture (Shih and Daigle, 1997). Starch, the principal component of rice, consists of 15 - 30% amylose and 70 - 85% amylopectin. Starch occurs in the endosperm as small many - sided granules, while protein is present as particles that lie between the starch granules (OCDE, 2004).



Figure 6. Rice grains in different forms

1.7. Fragrant rice

The rice varieties are classified in six groups based on the correspondences of isozyme variation at 15 loci among 1688 rice varieties, as shown in Figure 7 (Glazmann, 1987). Group I corresponds to the *indica* and Group VI to the *japonica*. Groups II, III, IV and V are atypical, but are also classified as *indicas* in the conventional classification. The largest group, Group I, is scattered all over Asia while Groups II, III, IV and V are discovered only on the Indian subcontinent, particularly in the foothills of Himalayas. Fragrant rice lies in Group I, V and VI. Group I includes Jasmine and some fragrant rice varieties from China, Vietnam and Cambodia. A limited number of cultivars belong to Group I (Indica) and Group VI (Japonica) and are considered non-fragrant. However, major cultivars belonging to Group V include in particular Basmati, a world famous high quality rice from Pakistan and as well as India. The center of origin of the Group V fragrant rice is the foothills of Himalayas in India where several fragrant rice cultivars are present. From the foothills, fragrant rice was introduced into several areas up to Myanmar region (Khush *et al.*, 2000).

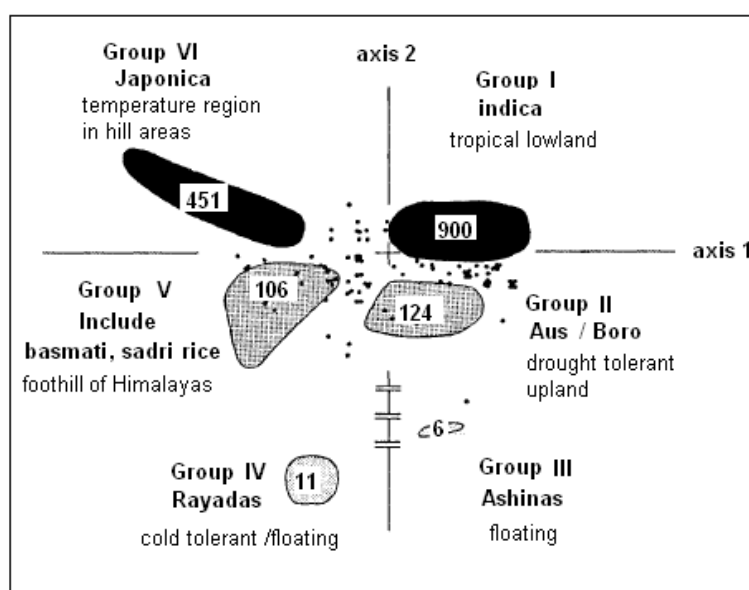


Figure 7. Six varietal groups on planes (1, 2) of a Factor Analysis of Correspondences of isozyme variation among 1688 rice varieties. Sizes of the groups are indicated. Isolated dots represent 90 varieties with intermediate positions or unstable classification (Glazmann, 1987)

1.7.1. Economical interest of production and consumption

Although fragrant rice varieties were cultivated originally in three countries (India, Pakistan, and Thailand), they are presently scattered throughout many countries in the world. But the total fragrant rice production, as well as exports, is insufficient for consumers globally. Basmati and Jasmine are premium long-grain rice due to their characteristic fragrance in both the raw and cooked state. They develop pleasant aroma and soft texture when cooking (Giraud *et al.*, 2010). The rice world market ranks fragrant rice at the top. Of the 4 - 5 million tons of fragrant rice worth 2 - 2.5 billion U.S. Dollars, Thai Jasmine rice shares almost 50% of the market with Basmati rice from India and Pakistan along with smaller rice producers such as the USA, Vietnam, and most recently Cambodia (Vanavichit, 2007). The price of the fragrant rice is usually 2 - 3 times higher than that of non fragrant rice. According to basmati.com, a web site that gives constant up to date Basmati market prices, the price of the Indian Basmati in March 2005 was \$850 USD per ton versus non fragrant rice varieties that has a range of \$238 to \$295 per ton, depending on the variety (Rondinone, 2007).

1.7.2. Environment and cultural practices

Many studies have reported grains present good aroma characteristics when rice has been cultivated in traditional fragrant rice areas (Rohilla *et al.*, 2000; Itani *et al.*, 2004; Yoshihashi *et al.*, 2004b; Gay *et al.*, 2006). Evaluation of the 2AP content, a potent aroma compound in fragrant rice, is considered a major factor of rice aroma quality. Although genetic traits is a main factor that can define quality of fragrant rice production, some environmental factors and cultivation practices can greatly affect its quality (Pinson, 1994, Gay *et al.*, 2010) such as temperature during grain filling and ripening (Itani *et al.*, 2004), soil type (Sagar and Ali, 1993) and timing of field drying and harvest (Arai and Itani, 2000; Champagne *et al.*, 2005). In Asian areas, fragrant rice grows better and produces the best quality grains under warm, humid, valley-like conditions (Giraud *et al.*, 2010). Fragrant rice obtained from irrigated areas in Thailand, contains lower 2AP levels than that from rainfed areas (Yoshihashi *et al.*, 2004b). Furthermore, both Basmati and Thai fragrant rice are harvested in the beginning of winter because of high aroma quality in rice grains (Lorieux *et al.*, 1996). In general, drought during the ripening stage, early harvest and cool climate conditions are preferred for production of fragrant rice with a strong aroma (Yoshihashi *et al.*, 2004a, 2004b; Itani *et al.*, 2004).

2. Soil salinity as the major environmental problem of plant

Salinity is a serious problem in several areas of the world and constitutes one of the major environmental factors that decrease agricultural productivity (Binzel and Reuveni, 1994). In saline soil, there are many environmental factors which responds to salt contamination, such as soil pH (acidic or alkaline), water and nutrient deficiencies that can lead to inhibit the growth and development of higher plants (James *et al.*, 2005; Kopittke and Menzies, 2005; Moradi and Ismail, 2007; Shi and Wang, 2005; Yang *et al.*, 2008). Saline soils are defined as loose and sandy soils with high amounts of water-soluble salts (e.g. sodium, chloride, calcium, magnesium and sulphate) and have an electrical conductivity (EC) higher than $4000 \mu\text{S.cm}^{-1}$, an exchangeable sodium percentage (ESP) lower than 15% and a pH lower than 8.5 (Rengasamy, 2002). Nowadays, field salinization is still expanding due to both natural processes and the mismanagement of irrigation. One-third of the world's food comes from irrigated land (Munns, 2002), thus salinization of agricultural soils is a critical issue.

2.1. Effect of salt stress on plant

Salt stresses can affect differently on plant growth based on the level of salt concentration as well as electrical conductivity (EC). According to the FAO (Food and Agriculture Organization), salinity could be divided into five levels, which are shown in **Table 2**. This data explained that plant growth is not affected from salt stress when EC values are less than $2000 \mu\text{S.cm}^{-1}$. In contrast, higher EC values showed increasingly damaging effects to plants. Salinity affects plants in different ways such as osmotic potential of soil solution (water stress), specific-ion toxicity (salt stress) and/or nutritional imbalance (Läuchli and Epstein, 1990). Salinity leads to adjusting metabolic processes in growing plants and involves changes in the expression of enzymes (Dubey, 1994; Richharia *et al.*, 1997; Khan and Panda, 2008).

Table 2. Indicative soil salinity - classes and implications for crop performances (adapted from FAO, 2001).

EC ($\mu\text{S.cm}^{-1}$) at 25 °C	Salt Concentration (mM)	Effect on crops
<2000	<20	mostly negligible
2000 - 4000	20 - 40	some damage to sensitive crops
4000 - 8000	40 - 80	serious damage to most crops
8000 - 15000	80 - 150	only tolerant crops succeed
> 15000	> 150	few crops survive

2.1.1. Effects of salt stress on physiological activities

The physiological mechanisms of crop plants development are affected by salinity stress. The concept of the two-phase growth (**Figure 8**) can explain inhibition of plant growth by salinity (Munns, 2002 and 2005).

The initial phase: salt outside the roots reduces the ability of the plant to take in water, which in turn, decreases leaf expansion. This effect leads to slower growth as the result of salt toxicity in leaves for both salt sensitive and salt tolerant plants (**Figure 8**). This condition is the osmotic or water-deficit effect of salinity. The growth reduction is presumably regulated by hormonal signals generated by the roots.

The second phase: response to growth reduction results from the toxic effects of salt inside the plant. The salt taken up by the plant concentrates in old leaves. Then salt is transported into transpiring leaves over a longer period leading to very high Na^+ and Cl^- concentrations until eventually the leaves die. This condition is salt specific or ion-excess effect of salinity. Salt sensitive genotype differs from a more salt tolerant one by its inability to prevent salt accumulation in leaves to toxic levels (Munns *et al.*, 2006).

Based on this concept, it can be assumed that primary response to salt stress may cause an ion imbalance as well as hyperosmotic stresses, and oxidative damage may occur in secondary stresses (Amirjani, 2011).

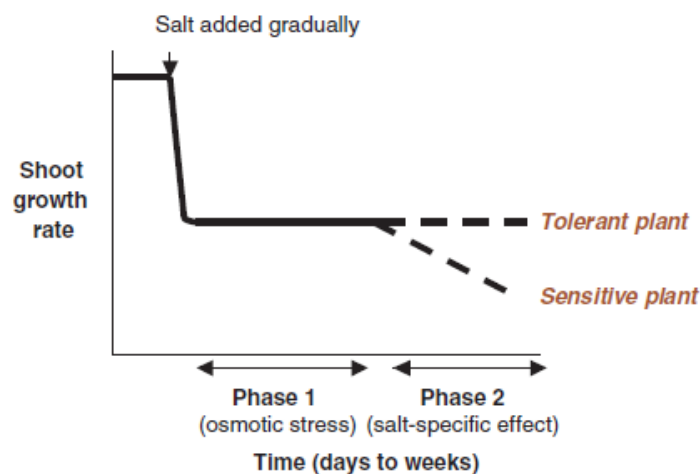


Figure 8. The two-phase growth response to salinity between salt tolerant and sensitive plants (Munns, 2005)

Salt toxicity can result in the death of leaves and also reduce the total photosynthetic leaf area. Reduced photosynthesis with high salinity is attributed to either stomatal closure, trending to a reduction in intracellular CO₂ partial pressure, or non-stomatal factors (Bethke and Drew, 1992). Salinity changes the photosynthetic parameters, including osmotic and leaf water potential, leaf temperature, transpiration rate, and relative leaf water content (RWC). Salt also affects photosynthetic components such as enzymes, chlorophylls, and carotenoids. Changes in these parameters depend on the severity and duration of stress (Misra *et al.*, 1997) on plant species (Dubey, 1994). In woody perennials such as citrus and grapevines, Na⁺ is confined within the woody roots and stems, whereas, Cl⁻ accumulates in the leaves and is most damaging to these plants most often by inhibiting the process of photosynthesis (Flowers, 1988).

2.1.2. Symptoms of salt stress of plants

Under salt stress, significant Na⁺ accumulation in plant tissues leads to injurious effects such as an imbalance in intracellular ionic concentrations (Greenway and Munns, 1980; Hu and Schmidhalter, 1998), oxidative stress (Foyer *et al.*, 1991; Borsani *et al.*, 2001), and inhibition of the photosynthesis process (Greenway and Munns, 1980; Shabala *et al.*, 2005). Most plants suffer from salt injury when electric conductivity (EC) values is in excess 4000 $\mu\text{S.cm}^{-1}$, while tolerant crops can resist much higher concentrations. The degree of salt injury depends on many factors such as the salt concentration, pH, temperature, air humidity, solar radiation, water depth, duration of exposure, and the crop growth stage (Levitt, 1980; Akbar and Ponnampereuma, 1982). Very high salt stress conditions damages the plant, but the moderate to low salt stress affects the plant growth rate and thus producing visible symptoms which could be associated with morphological, physiological or biochemical modifications. Salt accumulation in the expanding leaves has been correlated with photosynthetic reduction as well as ultra-structural and metabolic damages and sequential death of leaves (Yeo and Flowers, 1986). Salt injury starts with the reduction in effective leaf area. The oldest leaves start to roll then die, followed by the next older, and so on. Finally, the survivors have the old leaves losing vitality with the youngest remaining green. For rice, soil salinity above EC $\sim 3000 \mu\text{S.cm}^{-1}$ is moderate salinity while more than $9000 \mu\text{S.cm}^{-1}$ becomes very high (**Table 3**). Most of the parameters like low tillering, spikelet sterility, less florets per panicle, low 1000 grain weight and leaf scorching, are effects caused by salinity.

Table 3 Symptoms of rice salt injury at increasing levels of salinity
(Bonilla *et al.*, 2001)

Level of salinity stress	EC ($\mu\text{S}\cdot\text{cm}^{-1}$)	Symptoms
Medium salinity	3000 - 4500	nearly normal growth; some reduction in tillering; some leaves are roll
High salinity	5000 - 8500	reduced growth and tillering; most leaves are rolled; very few are elongating
Very high salinity	9000 - 14500	growth completely ceased; most leaves get dry and others are drying; in extreme cases, almost all plants withered

2.1.3. Effects of salt stress on plant growth stage and yield components

Most crop plants are salt tolerant at germination, but are salt sensitive during emergence and early vegetative development. Root and shoot growth is inhibited by salinity (Läuchli and Epstein, 1990; Maas and Grattan, 1999). The reproductive stage is considered less salt sensitive than vegetative growth, although in wheat salt stress can hasten reproductive growth, reduce spike development and decrease the yield potential. On the other hand, higher levels of salt stress leads to low yield, which is primarily associated with reduction in tillers and fertile florets in some cultivars. As plants mature, they become gradually more tolerant to salinity, in particular during the later stages. In general, salinity reduces shoot growth more than root growth (Läuchli and Epstein, 1990). It can reduce the number of fertile florets and increase sterility that cause loss of grain yield and affect the time of flowering and maturity in both wheat (Maas and Poss, 1989) and rice (Khatun *et al.*, 1995). Plants undergo characteristic changes from the time salinity stress is imposed until they reach maturity (Munns, 2002). Furthermore, the time from plantlet to maturity in cereal crops typically decreases with increased salinity, (Grieve *et al.*, 1993) but salinity has just the opposite effect on rice (Khatun *et. al*, 1995; Lutts *et al.*, 1995).

In one study of the effect of salt stress on M-202, a variety of rice, Zeng and Shannon (2000) reported linear decreases in several yield components with increased salinity including the percent of sterile florets, tillers per plant and spikelets per panicle, which translated into greater loss of grain weight per plant with increased intensity of salinity stress (**Figure 9**). However, they suggested that rice is most sensitive to salinity during the early vegetative growth, in particular, during the 3-leaf to panicle stage. Generally, salinity's effect on rice resulted in delayed flowering, a decrease in the number of productive tillers and fertile florets

per panicle and a reduction in individual grain weight (Khatun *et al.*, 1995; Lutts *et al.*, 1995). Furthermore, rice response to salinity is a combination of the level of salinity, the duration of exposure and the timing of exposure.

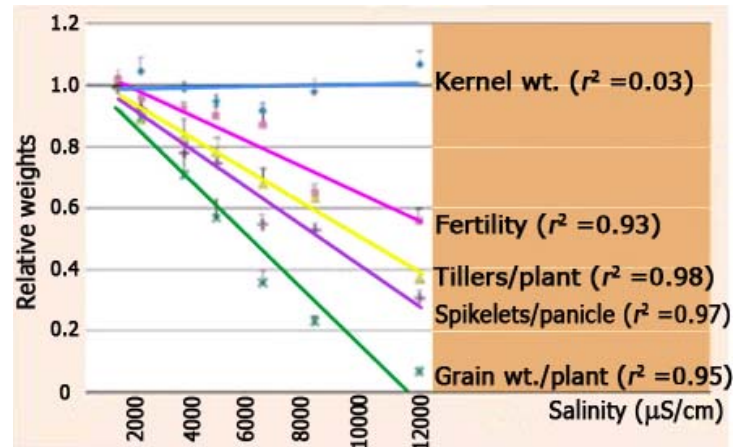


Figure 9. Relationship between salinity and various yield components of rice (*Oryza sativa* L. cv M-202). (Adapted from Zeng and Shannon, 2000)

2.2. Osmotic stress

The osmotic effect resulting from salinity may cause disturbances in the water balance of the plant and inhibit growth, as well as provoke stomatal closure and reduce photosynthesis (Hernandez and Almansa, 2002). Plants respond by means of osmotic adjustment, normally by inducing the concentrations of Na^+ and Cl^- uptake and compartmentalizing within their tissues, while synthesizing the organic compatible solutes such as proline, betaine, polyols and soluble sugars (Shimose, 1995). The three main types of osmoprotectant compounds (amino acids, ammonium compounds, and non-reducing sugars) are synthesized and accumulated in plants to lower the osmotic potential in the cytoplasm (Yancey *et al.*, 1982).

Such accumulation of inorganic ions may produce significant toxic effects, cell damage and deactivate both photosynthetic and respiratory electron transports (Amirjani, 2011). Using ions for osmotic adjustments may be energetically more desirable than organic osmolyte synthesis under stress (Raven, 1985). Nevertheless, the most common approach to osmotic adaptation, the change in osmotic potential, is the accumulation of compatible solutes, which plays an important role in protein and membrane stabilization.

2.3. Adaptation of plants to salinity stress

The ability of plants to tolerate salts is demonstrated through many metabolic pathways that simplify retention of water, protect chloroplast functions and maintain ion homeostasis (Parvaiz and Satyawati, 2008). Salt tolerance is a combination of different adaptations and regulations (Gorham, 1995), as in the synthesis of such organic solutes as sugars, proline and glycinebetaine (Greenway and Munns, 1980; Grumet *et al.*, 1985); translocation of Na^+ out of the leaves into phloem (Ren *et al.*, 2005); scavenging of free radicals and toxic compounds (Gueta-Dahan *et al.*, 1997; Yamamoto *et al.*, 2005); and secretion of salt onto the surface of the leaf through specialised salt glands (Tester and Davenport, 2003).

Halophytes may be able to survive under toxic saline levels through a balancing of the requirements of salt for maintaining osmotic balance and the essential metabolic processes for growth and development (Flowers and Flowers, 2005; Flowers *et al.*, 1986). Balancing may be imposed with reduced transpiration hence less salts in the shoots, including reduced leaf surface area and more essentially, salt glands (Thomson *et al.*, 1988) in the leaves to excrete excess salts.

2.3.1. Macromolecular homeostasis

Osmotic balance in plant cytoplasm during salt stress is maintained by increasing the levels of compatible organic solutes (osmolytes) and by accumulating excess salts in vacuoles (Serrano and Gaxiola, 1994; Hasegawa *et al.*, 2000). Although some compatible osmolytes are essential elemental ions, such as K^+ , the majority are organic solutes (Yokoi *et al.*, 2002). These organic compounds consists of sugars, organic acids, and nitrogen containing compounds such as amino acids (proline), amides, amino acids, proteins and quaternary ammonium compounds (glycine betaine) (Ashraf and Harris, 2004). The important characteristic of those compounds are low molecular weight with neutral charge because they do not interfere with normal cellular physiology (Zhifang and Loescher, 2003). They only replace water in biochemical reactions. The functions of osmolytes are mainly the protection of structures and osmotic equilibrium (Hasegawa *et al.*, 2000).

2.3.2. Ion homeostasis

Maintaining ion homeostasis is critical for plants to resist high salinity stress. Under normal conditions, plant cells maintain high K^+ levels of about 100 to 200 mM in order to maintain normal metabolic reactions. K^+ also plays a role in controlling turgor. In contrast, Na^+ levels should be less than 1 mM in cytoplasm for normal functioning of cells. Salinity causes a drastic decrease in potassium content of salt-sensitive rice varieties leading to the low ratio of K/Na (Asch *et al.*, 2000). Under salt stress conditions, plant cells need to further maintain high K^+ and low Na^+ levels. In this case, any excess Na^+ needs to be extracted from the cell or sequestered into the vacuolar compartment and given long-distance transport to facilitate their metabolic functions (Reddy *et al.*, 1992; Iyengar and Reddy, 1996; Zhu, 2003). Cellular ion homeostasis under salinity is accomplished by three strategies (Zhu, 2001):

- 1) Exclusion of Na^+ from the cell by plasma membrane bound Na^+/H^+ antiporters or by limiting the Na^+ entry.
- 2) Utilization of Na^+ or osmotic adjustment by compartment of Na^+ into the vacuole through tonoplast Na^+/H^+ antiporters.
- 3) Na^+ secretion

For example, Na^+ uptake by the roots that are transported to the leaves must be extruded from the cells or compartmentalized in the vacuoles in order to avoid accumulating Na^+ which is toxic to cellular proteins in the cytoplasm. Central to this process is the vacuolar Na^+/H^+ antiporter, which Na^+ exchange for H^+ across a membrane, and takes advantage of the proton gradient formed by these pumps (Mansour, 2003). Salt stress demonstrates increase of Na^+/H^+ activity in both glycophytes and halophytes (Apse and Blumwald, 2002). The activation of anti-porters will most likely operate in order to reduce the sodium toxicity in salt.

2.4. Role of proline during stress conditions

Proline is an amino acid and acts as a compatible osmolyte during salt stress in plants. It is not charged at neutral pH and is highly soluble in water. In responses to abiotic stresses i.e., salinity and water deficit in higher plants, this compound can be produced in high amounts (Hsu *et al.*, 2003; Kavi Kishor *et al.*, 2005; Ashraf and Foolad, 2007). In addition, salt stressed plants osmotic potential of vacuole is decreased by proline accumulation (Yoshida *et al.*, 1997). This does not inhibit biochemical reactions and plays a role as an

osmoprotectant during osmotic stress (Yoshida *et al.*, 1997). There are several possible roles have been attributed to supraoptimal levels of proline osmoregulation under drought and salinity conditions, stabilization of proteins, prevention of heat denaturation of enzymes, free radical scavengers and antioxidants (Sharma and Dietz, 2006) and conservation of nitrogen and energy for a post-stress period (Aloni and Rosenshtein, 1984).

In higher plants, proline is synthesized by either the glutamate or the ornithine pathway. The former is considered the major route, especially under osmotic stress (Delauney and Verma, 1993; Kavi Kishor *et al.*, 1995). Stimulation of proline synthesis in salt-stressed plants is correlated with increased Δ^1 -pyrroline-5-carboxylate reductase (P5CR) activity of halophytes (Treichel, 1986). Proline is usually synthesized from glutamate via Δ^1 -pyrroline-5-carboxylate (P5C) by two successive reductions, which are catalyzed by Δ^1 -pyrroline-5-carboxylate synthetase (P5CS) and P5CR (Hare *et al.*, 1999) (**Figure 10**). The first step is catalyzed by P5CS, and is a rate-limiting enzyme for the biosynthetic pathway in higher plants, being feedback inhibited by proline (Zhang *et al.*, 1995). On the other hand, proline catabolism is inhibited under osmotic stress (Peng and Verma, 1996; Kiyosue *et al.*, 1996). As the stress is removed, proline is oxidized to P5C by proline dehydrogenase. Then P5C is further converted back to glutamate by pyrroline-5-carboxylate dehydrogenase (P5CDH) (Verbruggen *et al.*, 1996).

The accumulation of proline under abiotic stress conditions accounts for fewer millimolar concentrations, depending on the species and the extent of stress (Delauney and Verma, 1993; Bohnert and Jensen, 1996). Very high accumulations of cellular proline (up to 80% of total of the amino acid under stress and 5% under normal conditions) is a result of increased synthesis and decreased degradation under stress conditions (Delauney and Verma, 1993; Kavi Kishor, 1988). In case of salt stress, several reports are mentioned on the activities of proline biosynthesis in plants as rapid salt defense responses such as in rice (Hien *et al.*, 2003), wheat (Wang *et al.*, 2007), barley (Ueda *et al.*, 2007), green gram (Misra and Gupta, 2005), sugar beet (Ghoulam *et al.*, 2002), etc.

In general, there is a consensus that proline has a vital role in the adaptation of cells to osmotic stress. However, doubts still persist whether the accumulation of this amino acid provides adaptive advantage or it is only a consequence of changes in the metabolism due to stresses (Serraj and Sinclair, 2002). Particularly in the case of rice, proline accumulation

seems to be a symptom of injury rather than an indicator of salt tolerance (Garcia *et al.*, 1997).

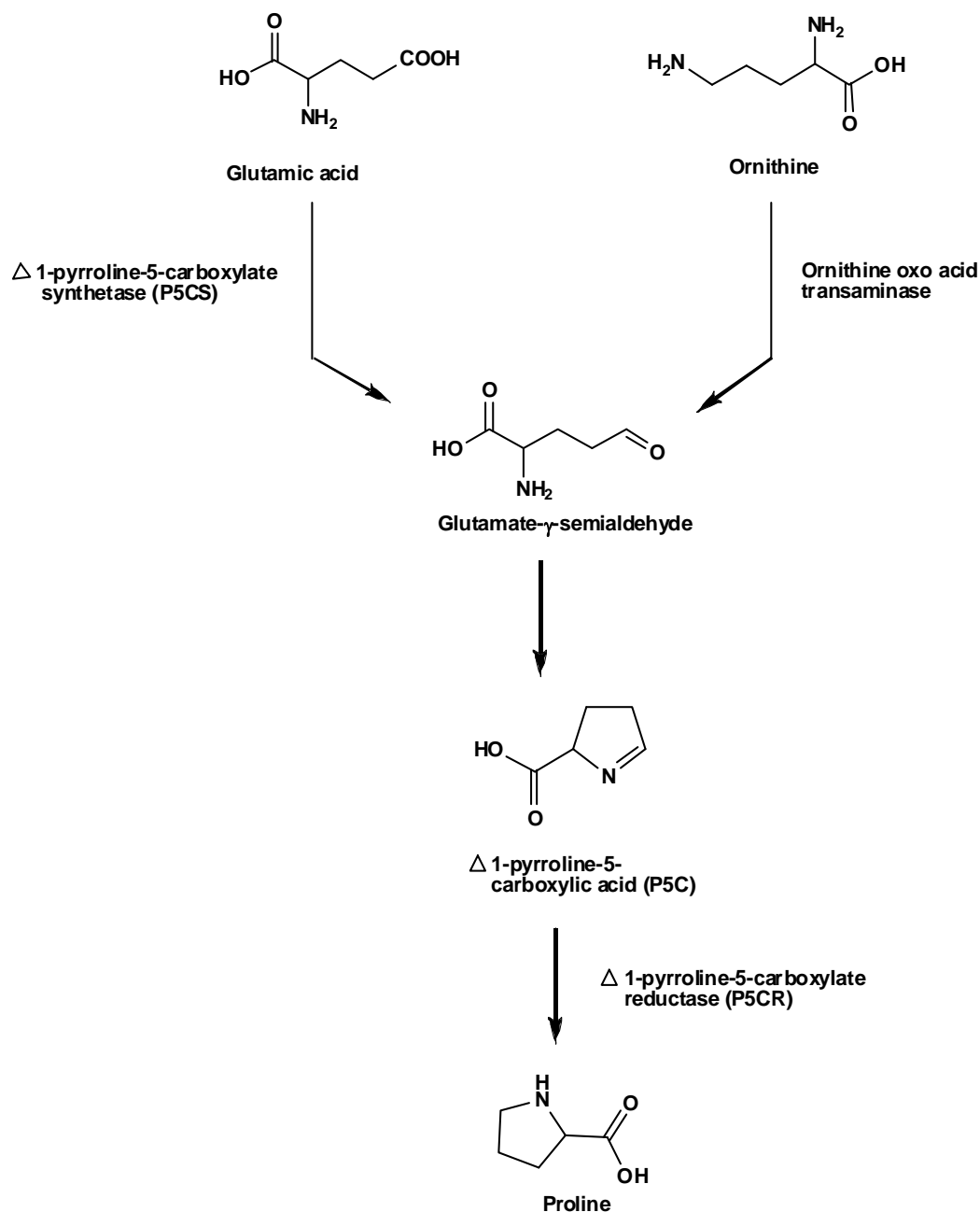


Figure 10. Biosynthetic pathway of proline (Adapted from Parvaiz and Satyawati, 2008)

2.5. Response of rice to salt stress

Rice growth and yield are severely affected by environmental stresses under arid and semi arid climates. Generally, most rice varieties are sensitive to salinity (Grover and Pental, 2003) however; some traditional indica rice varieties such as Pokkali, Nona Bokra and Kalara are fairly tolerant to salinity (Yeo *et al.*, 1990).

Rice is the only cereal that has been recommended as a desalinization crop due to its capability to grow well under submerged conditions. Moreover, the standing water in rice fields can help to leach salts from the topsoil to reduce salinity for subsequent crops (Bhumbla and Abrol, 1978). Due to the formation of an aerenchyma, rice can adapt better to flooding than most other annual crops, which typically cannot survive under submerged conditions for more than five days (White *et al.*, 1996).

In spite of its high sensitivity to salt stress, substantial genetic variations in salt tolerance have been observed among rice varieties at different developmental stages (Akbar *et al.*, 1972; Flowers and Yeo, 1981). Rice is highly sensitive to NaCl stress and even 0.05 M NaCl is damaging for rice during the early seedling stage. It has been further demonstrated that although tall rice varieties are low yielding, they are generally more salt tolerant compared with agronomically suitable semi dwarf varieties (Yeo *et al.*, 1990).

Rice is comparatively tolerant of salt stress during germination, active tillering, and during maturity, however, it does become salt sensitive during the early seedling and reproductive stages (Lafitte *et al.*, 2004). Moradi *et al.* (2003) suggested that salt tolerance at the seedling and reproductive stages is weakly associated; therefore, pyramiding significant traits in both stages is required for developing resilient salt tolerant rice cultivars.

3. Flavor of rice

3.1. Volatile compounds from rice

Over 300 volatile compounds have been identified from various cultivars of fragrant and non fragrant rice (Yajima *et al.*, 1979, Widjaja *et al.*, 1996, Maraval *et al.*, 2008). Most of the volatiles found in fragrant and non fragrant rice were similar. Weber *et al.* (2000) suggested that the pleasant odor of raw or cooked non fragrant or fragrant rice was controlled by a blend of various volatiles. Previous work found that the volatiles from traditional rice had higher amounts of 4-vinylphenol, 1-hexanol, and 1-hexanal, but lower amounts of indole than fragrant rice (Maga, 1984). Buttery *et al.* (1988) investigated the contribution of the several volatiles to the aroma of cooked California long-grain rice as the first systematic approach. Odor activity values (OAV) are calculated by dividing the concentrations by the odor thresholds. They confirm 2AP, (E,E)-deca-2,4-dienal, nonanal, hexanal, (E)-non-2-enal, octanal, decanal, 4-vinyl-2-methoxyphenol, and 4-vinylphenol with low odor thresholds as the most potent among the 64 odorants identified (Buttery *et al.*, 1988). In particular, 2AP as

the potent odorant contributor of fragrant rice (pop-corn attribute) can be used to differentiate between fragrant and non fragrant rice varieties (Buttery *et al.*, 1982). Some volatile compounds that contribute to the aroma of rice with their aroma descriptions from both fragrant and non fragrant rice varieties are shown in Table 4 (Maraval *et al.*, 2008).

Sunthonvit *et al.* (2005) reported that some volatiles in rice, i.e., aldehydes, ketones, alcohols and heterocyclic compounds could be formed by the thermal process.

Aldehydes are one of the major volatile compound classes found in rice grain. Some volatile aldehydes are formed from unsaturated fatty acids such as oleic, linoleic and linolenic acid by autoxidation (Houston, 1972). For example, hexanal in rice may arise from decomposition of linoleate 13-OOH and 9-OOH hydroperoxides.

Alcohols in rice samples can be formed through the decomposition of fatty acids through lipoxygenase activity. Hsieh (1994) proposed that 1-octen-3-ol was derived from the 10-hydroperoxide of linoleic acid.

Heterocyclic compounds could be generated through non-enzymatic browning reaction (the Maillard reaction). However Yoshisahi (2002a) has demonstrated that 2-acetyl-1-pyrroline in rice is mainly of biosynthetic origin. Cooking of rice yielded quite low levels of 2AP (Yoshiashi, 2002a).

Table 4. Volatile aroma compounds in fragrant and non-fragrant rice varieties (Maraval *et al.*, 2008)

Aroma compound	Odor Theshold ($\mu\text{g.L}^{-1}$) ^a	Odor description	Quantification ($\mu\text{g.kg}^{-1}$)			
			Aychade ^c	Fidji ^c	Thai ^c	Ruille ^d
Hexanal	5	Green, grass	70	61	122	42
Butanol	500	Medicinal	39	45	116	74
Pentan-1-ol	4000	Sweet, strong	107	trace	343	7
Octanal	0.7	Citrus, fatty	50	40	44	40
(E)hept-2-enal	13	Fatty, green	275	176	58	64
6-methylhept-5-en-2-one	50	Herbal, green	26	59	29	39
2-acetyl-1-pyrroline	0.1	Sweet, popcorn	215	264	186	trace
Hexan-1-ol	2500	Sweet, green	59	43	35	19
Nonanal	1	Floral, fatty	167	152	188	134
(E)oct-2-enal	3	Green, herbal	48	trace	trace	trace
Oct-1-en-3-ol	1	Herbal, earthy	129	91	103	22
2-ethyl hexan-1-ol	270000 ^b	Oily, sweet	25	68	32	81
(E)non-2-enal	0.08	Fatty, waxy	trace	trace	trace	trace
Benzaldehyde	350	Nutty, sweet	47	38	99	80
(E,E)deca-2,4-dienal	0.07	Fatty, citrus, powerful	163	117	25	18
2-methyl-4-vinylphenol	3	clove, burnt	373	254	563	317
4-vinylphenol	10	phenolic, medicinal	1127	1079	trace	962
Indole	140	Tar, floral	252	108	69	356

^a odor theshold values in water from Buttery *et al.* (1988)^b odor theshold values in water from Fazzalari (1978)^c fragrant rice variety^d non fragrant ricetrace < 2 $\mu\text{g.kg}^{-1}$

3.1.1. 2-Acetyl-1-pyrroline, a potent volatile compound in rice

2-acetyl-1-pyrroline (2AP), with its popcorn-like aroma, has been identified as a key flavoring compound in fragrant rice (Buttery *et al.*, 1983b; Widjaja *et al.*, 1996). Many studies reported that non fragrant rice varieties contain very low levels of 2AP (less than 8 ppb) while its level in fragrant genotypes is much higher (from 40 to 900 ppb) (Buttery *et al.*, 1983b; Maraval *et al.*, 2008). Earlier studies reported that 2AP is the major flavour compound of

Pandanus leaves, and its level was 10 and 100 times more than that present in fragrant and non fragrant rice, respectively (Buttery *et al.*, 1983a). Among rice volatiles, 2AP possesses the lowest odor threshold, thus allowing humans to detect few concentrations in field-grown plants or crushed leaf tissue, as well as in the grain before and after cooking. Its odor threshold in the air is 0.02 ng.L⁻¹ (Schieberle, 1991). Its odor threshold in water is 0.1 µg.kg⁻¹ that makes easy to detect it in cooked rice (Buttery *et al.*, 1983b). 2AP was reported in other products : canned sweet corn (Buttery *et al.*, 1994), toasted wheat bread (Rychlik and Grosch, 1996), moderately roasted sesame (Schieberle *et al.*, 1996) and as well as in pandan (*Pandanus amaryllifolius* Roxb.) leaves as mentioned before.

3.2. Extraction and analysis of volatile compounds from rice

There are several techniques in extraction and analysis of volatile compounds. Here we report mainly techniques used for the extraction and analysis of 2AP from rice.

3.2.1. Extraction techniques for 2AP from rice

Liquid–solid extraction (LSE)

Liquid–solid extraction (LSE) is the most common technique used to extract analytes from the plant material using organic solvents. Solvent like dichloromethane was used to extract 2AP from the rice grains (Mahatheeranont *et al.*, 2001).

Simultaneous steam distillation/ solvent extraction (SDE)

2AP and other volatile components were extracted by simultaneous steam distillation/ solvent extraction (SDE) apparatus (Buttery *et al.*, 1983b) (Likens-Nickerson technique). One of the major disadvantages of this technique that it can lead to the formation of artifacts by degradation of some aroma precursors during the thermal extraction process.

Solvent Assisted Flavor Evaporation (SAFE)

Solvent assisted flavor evaporation (SAFE) was developed as a careful method for the isolation of volatiles under vacuum from solvent extracts, aqueous food suspensions or even matrices with high oil content. SAFE comes with diverse advantages such as avoidance of the formation of artifacts during the extraction process, higher yields of volatiles compared with previously used high vacuum transfer techniques, higher yields of more polar flavor substances and odorants from fat-containing matrices (Engel *et al.*, 1999). This technique was applied to extract aroma compounds from cooked rice (Jezussek *et al.*, 2002).

Direct thermal analysis technique

Direct thermal analysis technique allows the analysis of wide ranges of volatiles and semi-volatiles in different types of materials (Iqbal *et al.*, 2000). In this technique, volatiles can be thermally extracted directly and together with the carrier gas flow through a solid matrix sample. The volatiles are then desorbed in GC injection port for subsequent analysis via the GC/FID and/or GC/MS. This technique has been used to distinguish fragrant and non fragrant varieties (Iqbal *et al.*, 2000).

Headspace sampling techniques

Static headspace gas chromatography (SHS-GC) is a technique particularly suitable for separating and determining volatile compounds present in solid or liquid samples. The method is based on the analysis of the vapor phase in equilibrium with the solid or liquid phase. The possibility of artifact formation using this procedure is minimal because neither severe temperatures nor organic solvents are used. This method has advantages in that the sample preparation step and the total analysis time are much shorter and is fully automated and solvent-free. This technique has been applied to qualitative analysis of 2AP in grains of fragrant rice (Sriseadka *et al.*, 2006)

Solid phase microextraction (SPME), a mild extraction method has proven to be a simple, rapid and sensitive method for collecting the volatile compounds from the headspace of a sample (Belardi and Pawliszyn, 1989). Previous work has found that recoveries of 2AP from the headspace of rice showed a quite low recovery factor $< 0.3\%$ by the SPME (Wongpornchai *et al.*, 2003). On the contrary, a recent work demonstrated that the recovery of 2AP from the rice matrix by SPME was almost complete (Maraval *et al.*, 2010). Stable isotope dilution analysis was developed in the latter case.

3.2.2. Quantification of 2AP in rice

After extracting the volatile compounds from the sample, semi-quantification of 2AP compound was conducted using 2,4,6-trimethylpyridine (TMP) or collidine (trivial name) as internal standard through gas chromatography or gas chromatography-mass spectrometry (GC-MS) analysis (Buttery *et al.*, 1986). Yoshihashi (2002a) developed a stable isotope dilution assay (SIDA) technique to quantify precisely 2AP amount in rice leaves and grains through GC-MS-SIM analysis. Satisfactory results were provided discarding the matrix effect. The recovery was linear from 5 to 5000 ng.g⁻¹ with a sensitivity at least 0.1 ng.g⁻¹ for 2AP.

A rapid method employing static headspace gas chromatography (SHS-GC) (Sriseadka *et al.*, 2006) has been developed and validated for quantitative analysis of 2AP in grains of fragrant rice. This method provides satisfactory recovery and sensitivity of 2AP by the utilization of both flame ionization detector (FID) and nitrogen-phosphorus detector (NPD). Additionally, SHS-GC-NPD demonstrated the possibility of both higher selectivity and sensitivity of the analysis of 2AP to be as low as 5 ng. Recently, a stable isotope dilution assay (SIDA), involving headspace solid-phase microextraction (HS-SPME) combined with gas chromatography–positive chemical ionization-ion trap-tandem mass spectrometry (GC–PCI-ITMS–MS) was developed for 2AP quantification in fragrant and non fragrant rice plant tissues and grains (Maraval *et al.*, 2010). Limits of detection (LOD) and quantification (LOQ) for 2AP were 0.1 and 0.4 ng.g⁻¹ of rice, respectively. Low amounts of rice samples, less than 1 g can be used for the quantification of 2AP in a rapid, accurate and sensitive analysis.

3.3. Biological 2AP formation pathway

3.3.1. Studies of 2AP biosynthesis : source of pyrroline ring

The biological formation of 2AP was first demonstrated on metabolites of *Bacillus cereus* isolated from cocoa fermented in the presence of proline and ornithine (Romanczyk *et al.*, 1995). The authors hypothesized that the contamination of rice plants by this bacterium may play a role in the formation of 2AP. However in disinfected rice, the presence of 2AP has been demonstrated (Yoshihashi *et al.*, 2002b). Thus the hypothesis of a microbiological origin was excluded and the hypothesis of a synthesis by the plant itself is now obviously accepted. The biosynthesis of 2AP in rice was initially studied in the callus of Basmati fragrant rice by using proline as a possible precursor (Suprasanna *et al.*, 1998). Addition of proline into rice callus growth medium induced overproduction of 2AP. Yoshihashi *et al.* (2002b) tested the feeding impact of seven amino acids on 2AP synthesis in rice callus and seedlings. Significant increases in 2AP concentration were observed with three amino acids fed, mainly proline, and to a lesser extent ornithine and glutamate. By feeding rice callus with ¹⁵N-labeled and ¹³C-labeled proline, it was demonstrated that ¹⁵N from proline was incorporated into pyrroline ring but carboxyl group of proline was not found in acetyl group of 2AP (Yoshihashi *et al.*, 2002b) (**Figure 11**). Recently it has been proposed that 1- pyrroline may derive from Δ1-pyrroline-5-carboxylate through glutamic acid (Huang *et al.*, 2008).

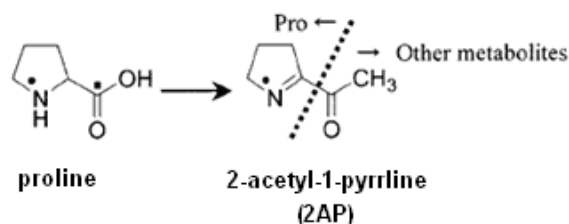


Figure 11. Formation of 2-acetyl-1-pyrroline in rice by C13-labeled proline as precursor (Yoshihashi *et al.*, 2002b)

3.3.2. Studies of 2AP biosynthesis : source of acetyl group

Romanczyk *et al.* (1995) demonstrated that *Bacillus cereus*, isolated from cocoa fermentation can generate ¹³C-labeled 2AP compound by using ¹³C-labeled glucose. Their results indicated that two atoms of ¹³-C from labeled glucose were incorporated within 2AP in the acetyl carbon chain. Costello and Henschke (2002) further suggested that the acetyl group of 2AP can be derived from fructose when either ethanol or acetaldehyde is supplied to *Lactobacillus hilgardii* isolated from wine. Moreover, Adams and De Kimpe, 2007 suggested that the biological synthesis of 2AP by *B. cereus* was probably done through acetylation of 1-pyrroline issued from two amino acids, ornithine and proline (**Figure 12**). In addition, they also proposed that acetyl CoA may be a potential candidate of acetyl donor on 2AP biosynthesis pathway. This is in agreement with 2AP synthesis when acetyl CoA was used as a precursor in a rice callus culture from a non basmati rice cultivar (Suprasanna *et al.*, 1998).

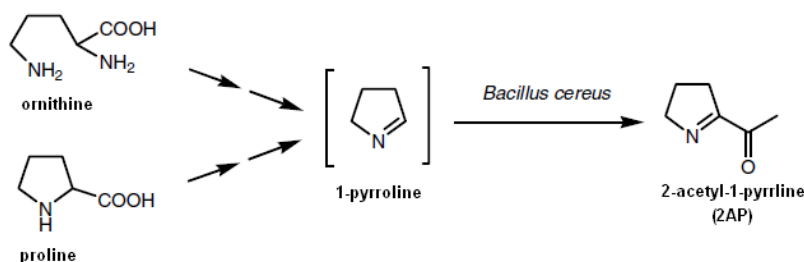


Figure 12. Formation of 2-acetyl-1-pyrroline from ornithine and proline by *Bacillus cereus* (Adams and De Kimpe, 2007)

3.3.3. 2AP biosynthesis pathway in rice

Two biosynthesis pathways of 2AP in rice have been proposed (**Figure 13 and 15**). Proline was transformed metabolically into γ -aminobutyraldehyde (GABald) via oxidation of putrescine (Bradbury *et al.*, 2008) (**Figure 13**). GABald is substrate of betaine aldehyde dehydrogenase (*BADH2*). Bradbury *et al.* (2008) suggested that there are two possibilities with regard to *BADH2* activity in the biosynthesis pathway of 2AP. If *BADH2* is functional

(case of non fragrant varieties) GABald is oxidized to γ -aminobutyric acid (GABA). On the other hand, if *BADH2* is non-functional (case of fragrant varieties) the GABald can spontaneously cyclise into 1-pyrroline. The latter is then acetylated to generate 2AP (Bradbury *et al.*, 2008). Vanavichit *et al.* (2005) has suggested the same pathway. 1-pyrroline appears then as a major intermediate in the accumulation of 2AP in fragrant rice varieties in contrast to non fragrant rice varieties.

Bradbury *et al.* (2008) established a close relation between 2AP synthesis and specific genes. Genetic studies showed that synthesis is controlled by recessive allele of the fragrance gene (*fgr*) mapped on chromosome 8 (Lorieux *et al.*, 1996; Jin *et al.*, 2003). **Figure 14** shows an eight base pairs (8-bp) deletion in the exon 7 of this gene resulting in truncation of betaine aldehyde dehydrogenase (*BADH*) whose loss of function lead to the accumulation of 2AP in fragrant rice (Bradbury *et al.*, 2005). Fitzgerald *et al.* (2008a) suggested that the 8-bp deletion in the *fgr* gene is not the only cause of 2AP synthesis that is controlled by at least one other mutation in a second locus.

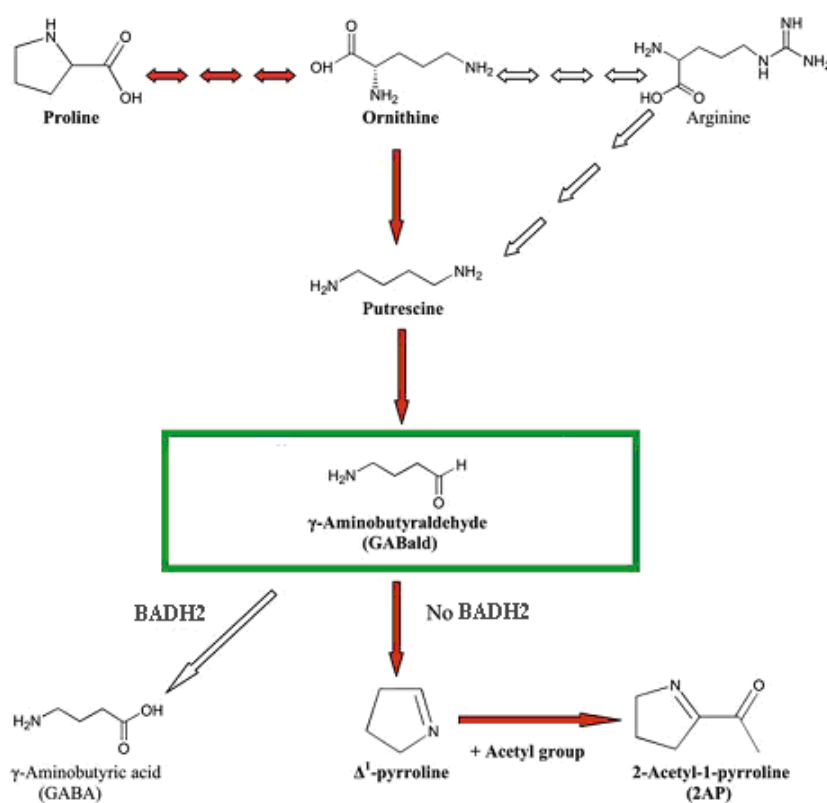


Figure 13. Proposed biosynthesis pathway of 2AP in rice by *BADH2*-dependant 2AP synthesis (Bradbury *et al.*, 2008).

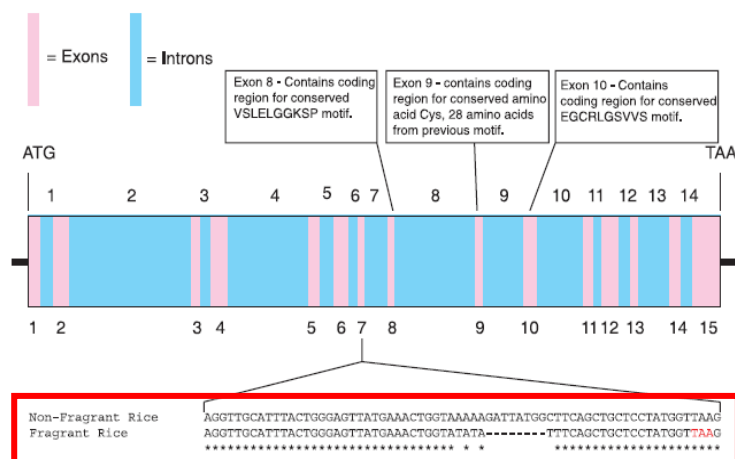


Figure 14. Structure of the fragrance gene (*fgr*) comparing the nucleotide sequence of exon 7 as shown for both non-fragrant and fragrant rice varieties (Bradbury *et al.*, 2005)

In the second pathway 1-pyrroline is derived from 1-pyrroline-5-carboxylate (P5C) (**Figure 15**). The latter was synthesized by Δ 1-pyrroline-5-carboxylate synthetase (P5CS) which is more abundant in fragrant than non-fragrant rice varieties (Huang *et al.*, 2008). It has been proposed that 2AP in fragrant rice is generated by a direct reaction between 1-pyrroline and methylglyoxal (Huang *et al.*, 2008).

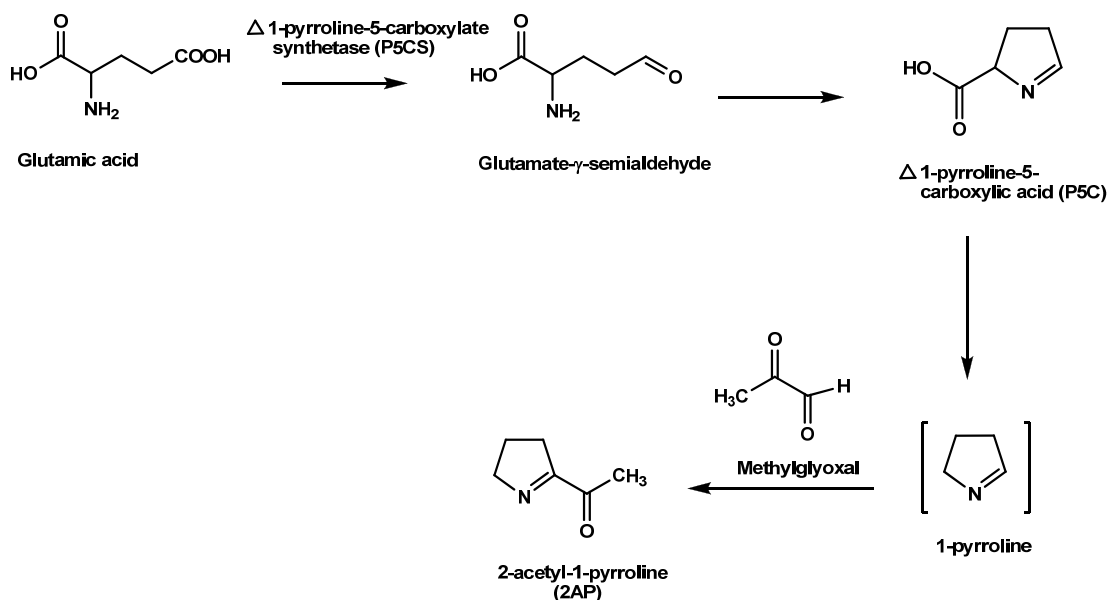


Figure 15. Proposed biosynthesis pathway of 2AP in rice (Huang *et al.*, 2008)

3.3.4. 2AP biosynthesis in rice under stress conditions

2AP concentration depends on genotype of rice varieties as Bradbury *et al.* (2005) describes, however, the concentration of 2AP in rice is controlled not only by genetic factors, but also by environmental parameters or cultivation practices. Previous studies support the hypothesis that osmotic stress, i.e. drought and salinity, may have a positive response on aromatic quality of fragrant rice. In the field, Yoshihashi *et al.* (2004a) suggested that drought stress during the ripening phase is provided to elevate 2AP content in the grains at harvest. During osmotic stress such as drought and salt, a high level of proline is biosynthesized via intermediate Δ^1 -pyrroline-5-carboxylic acid by both glutamate and ornithine pathways (Kavi Kishor *et al.*, 2005). This may lead to enhance 2AP synthesis in fragrant rice because of higher levels of proline. However the work of Fitzgerald *et al.* (2008b) reported no significant correlation between 2AP levels in fragrant rice leaves from Jasmine and Basmati rice varieties and salt stress during the vegetative stage. Recently, Gay *et al.* (2010) however found that salinity stress before flowering results in increasing proline content in the rice plant, and then alternately increasing 2AP biosynthesis in the leaves and grains. Thus they proposed that 2AP content of fragrant rice grains could be governed by salt stress in the field.

Part 1

Biosynthesis of 2AP in rice plant under controlled salinity conditions

Environmental factors such as temperature during grain filling and ripening (Itani *et al.*, 2004), soil type (Sagar and Ali, 1993) and soil salinity (Pinson, 1994, Gay *et al.*, 2010) can greatly affect rice aroma and rice production. Soil salinity is one of the main environmental factors leading to decrease in plant productivity. Although rice can be cultivated in saline soil, a large number of rice varieties, in particular fragrant varieties are sensitive to salinity (Grover and Pental, 2003). Rice plant is affected by soil salinity mainly during the early seedling and reproductive stages (Heenan *et al.*, 1988; Lutts *et al.*, 1995). It has been reported that symptoms of salinity appears when electrical conductivity (EC) of the soil is higher than 2000 $\mu\text{S}\cdot\text{cm}^{-1}$ (Asch and Wopereis, 2001; Grattan *et al.*, 2002). Saline conditions can affect development of rice plants in such ways as delayed flowering, a decline in the number of tillers and fertile florets per panicle and a reduction in individual grain weight thus reduction in crop productivity (Khatun *et al.*, 1995; Lutts *et al.*, 1995; Zheng and Shannon, 2001). Besides drought during ripening and harvest during cool climate conditions (Yoshihashi *et al.*, 2004a; Itani *et al.*, 2004), salinity of the soil has been found to favor the synthesis of 2-acetyl-1-pyrroline (2AP), a characteristic flavoring compound of fragrant rice (Gay *et al.*, 2010). However no relationship was found between 2AP levels in the leaves from two fragrant varieties and salt treatment during experiments in a glasshouse (Fitzgerald *et al.*, 2008b). Scientific data is quite scarce for understanding biosynthesis of 2AP under salt stress. On the other hand proline, the main amino acid precursor of 2AP (Yoshihashi *et al.*, 2002b) was found to accumulate during osmotic stress such as drought and salt (Kishor, *et al.*, 2005). Consequently it appeared plausible that this increase may induce 2AP biosynthesis. However, reports on the relation between proline and 2AP during salt stress almost inexistent except for a recent paper (Gay *et al.*, 2010).

This part focuses on the biosynthesis of 2AP in relation with proline as well γ -aminobutyric acid (GABA) under controlled salt treatment. GABA was also targeted compound since it is an important metabolite in the biosynthesis pathway of 2AP. Moreover its synthesis is often enhanced under salt stress (Kim *et al.*, 2007).

This part reports the results of two experiments. A fragrant rice variety (Aychade) was used in both cases. The first experiment was conducted in a simple model consisting of seedlings culture under different levels of salt (NaCl). 2AP, proline and GABA levels together with seedling growth parameters were determined. A paper entitled

“Salt stress during rice seedling culture induces 2-acetyl-1-pyrroline biosynthesis, a dominant flavor compound in fragrant rice”.

which was submitted in november 2011 to the journal of “ *industrial crops and products*”.

The second experiment was performed in the pots filled with the soil in a greenhouse. The effect of salt treatment during different growth stages of rice on the biosynthesis of 2AP, proline and GABA was investigated. A paper will be submitted in the following months to the journal of “Journal of Agricultural and Food Chemistry”:

“Effect of timing and duration of salt treatment during growth of a fragrant rice variety on 2-acetyl-1-pyrroline biosynthesis, key flavour compound in rice”

In the following pages prior to the presentation of two papers above, experimental setup and analytical methodology will be presented.

Part 1

Materials and Methods

Experiment 1 : Biosynthesis of 2AP in rice seedlings under different levels of salt solution

1.1. Plant material

Rice (*Oryza sativa* L.) seeds (harvest 2009) were from Aychade, a fragrant rice variety cultivated in Camargue area in South of France.

1.2. Seedling culture experiment

1.2.1. Liquid medium

Half-strength MS solution (Duchefa, Netherlands) (Murashige and Skoog, 1962) was prepared from MS (4.6 g.L⁻¹ of distilled water) and autoclaved at 121°C, 20 min prior to use for rice seedling culture. The composition of MS medium is given in **Table 5**.

Table 5: Composition of MS medium. (Murashige and Skoog, 1962)

Constituent	Concentration (mg/L)
Microelements	
NH ₄ NO ₃	1650
KNO ₃	1900
MgSO ₄ .7H ₂ O	370
CaCl ₂ .2H ₂ O	440
KH ₂ PO ₄	170
Microelements	
H ₃ BO ₃	6.2
MnSO ₄ .4H ₂ O	22.3
ZnSO ₄ .7H ₂ O	8.6
KI	0.83
Na ₂ MoO ₄ .2H ₂ O	0.25
CuSO ₄ .5H ₂ O	0.025
CoCl ₂ .6H ₂ O	0.025
Fe.EDTA-Na salt	37.3
Vitamins	
Nicotinic acid	0.5
Thiamine HCl	0.1
Pyridoxine HCl	0.5
Glycine	2.0
Myo-inositol	100

1.2.2. Seed culture and samplings

Rice seeds from Aychade were dehulled and sterilized in 70% (v/v) ethanol for 3 min following by shaking vigorously every 5 min for 50 min in a sodium hypochlorite solution (40%; v/v). The seeds were rinsed four times with sterilized distilled water then blotted on sterile Whatman filter paper sheets. Germination took place in Petri dishes on Whatman 3MM filter papers moistened with deionized and sterilized water. Germinated seeds were immersed in 8 mL of half-strength MS sterilized solution placed in Ø 9 cm Petri dishes (20 seeds per dish). The dishes were wrapped with Parafilm to reduce evaporation and contamination. All stages were carried out inside the laminar flow cabinet to protect seed against microbial contamination. Seedlings development took place in a growth chamber with a photoperiod of 14/10 h light/dark regime at 25°C. After 1 week, ninety homogeneous seedlings were transferred into 2 L Erlenmeyer flask added with 160 mL of MS solution (**Figure 16**). A total of fifteen Erlenmeyer flasks were prepared in order to have three replicates of each of subsequent five treatments: 0; 1.7; 17; 85 and 170 mM NaCl. Note that in preliminary work on seedling experiments, an effect of individual plant was observed on the levels of targeted compounds, i.e., 2AP, proline, GABA whatever salt level applied. Consequently each treatment was performed in triplicate and each replicate contained a high number of plants (ninety).

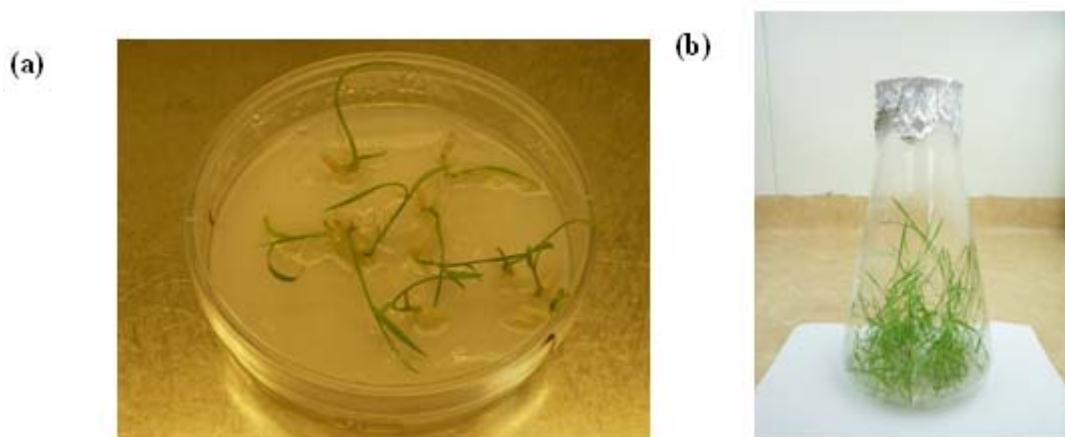


Figure 16 Rice (cv. Aychade) seed germination 5 days after culture (DAC) (a) and rice seedling 10 (DAC) (b)

After 2-weeks of growth, the MS medium from each Erlenmeyer flask was removed and replaced by MS solution containing 0; 1.7; 17; 85 and 170 mM NaCl, respectively (**Figure 17**). After 1 week of incubation, ninety seedling shoots from each Erlenmeyer flask were harvested (**Table 6**) and fresh weights were determined. Dry weight of the shoots was estimated as mentioned below using ca., 0.5 g of material. The remaining shoots representing

ca., from 9.0 to 10.80 g were ground under liquid nitrogen. The powdered samples were stored at -20°C until 2AP, proline and GABA analysis.

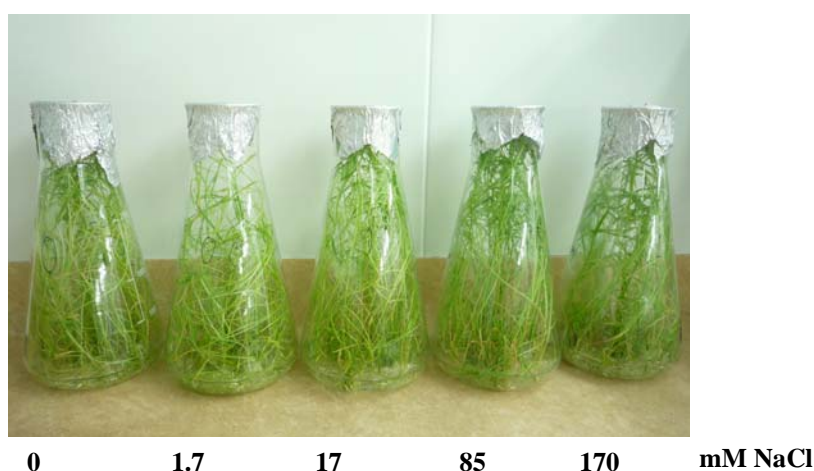


Figure 17 Rice (cv. Aychade) seedlings from five treatments 20 days after culture (DAC)

Table 6. Number of harvested rice seedlings from five treatments

NaCl (mM)	0	1.7	17	85	170
(1) Harvest (week)	3	3	3	3	3
(2) Number of replicates per treatment	3	3	3	3	3
(3) Number of plants per replicate	90	90	90	90	90
(4) Number of plants per treatment (2) x (3)	(270)	(270)	(270)	(270)	(270)

1.2.3. Determination of seedlings growth

Growth of seedlings was determined by measuring the changes in the fresh weight (FW) and dry weight (DW) of the shoots. 90 seedling shoots from each Erlenmeyer flask were weighed to obtain fresh weight. For dry weight measurement, shoots (0.5 g) were cut in small pieces and placed on aluminium bowl, and then dried at 70°C in an oven for 3 days until weight was constant (Gay *et al.*, 2010). Determinations of DW were done in duplicate and results were expressed as FW (g) and DW (g). The water content was expressed as gram of water per gram of dry weight of the shoots. The %water content of shoots was calculated as (Fresh weight - Dry weight) / Fresh weight x 100.

1.3. 2-Acetyl-1-pyrroline (2AP) analysis

See Experiment 2 in chapter 3.2 Conditions of 2AP analysis

1.4. Proline and GABA analysis

See Experiment 2 in chapter 4. Proline and GABA analysis

1.5. Statistical analysis

The data was subjected to analysis of variance (ANOVA) with mixed models, as well as to regression analysis ($p < 0.05$) using XLSTAT Pro 2010 software (Addinsoft, France).

Experiment 2: Biosynthesis of 2AP in rice plant under different salt treatments in a greenhouse

2.1. Plant material

Rice seeds were from Aychade harvested in Camargue field in 2009.

2.2. Greenhouse experiment

The experiments were conducted in a greenhouse of Centre de Coopération Internationale en Recherche Agronomique pour le Développement (Cirad) in Montpellier (France). Seed were sown on June 3 in 2010. Harvest happened on October 4 in 2010 (124 Days after sowing; DAS).

2.2.1. Soil for rice cultivation

Soil was prepared from mixture of fertile soil (from Lavalette field, Montpellier, France) (75%) and natural compost (25%), a blend of decomposed remains of peat mosses (*Sphagnum sp.*) and herbs. 330 pots were prepared. Each pot of 2 L capacity (height of ca., 20 cm) was filled with 1500 g of the prepared soil and 4g of a chemical fertilizer (Basacote Plus 6M, trade name; N:P:K ratio of 11:9:19).

Main physicochemical characteristic of the soil from three periods; before sowing, before the starting salinity stress at beginning tillering (BT) stage and after the last salinity stress at flowering (FLO) stage were given in **Table 7**. Higher level of organic compounds in soil from BT and FLO stages is due to the use of chemical fertilizer.

Table 7. Main physicochemical characteristics of the soil from three different periods during rice growth

Characteristic	Before sowing	BT stage (T0)	FLO stage (T0)
Organic matter (%)	5.95	7.24	7.14
Organic carbon (%)	3.45	4.20	4.14
Total nitrogen (%)	1.39	1.71	1.59
C:N ratio	24.80	24.61	25.96
Cation exchange capacity (me/100g)	11.47	14.02	13.87
Saturation rate (%)	> 100	> 100	> 100
pH	8.01	7.86	7.86

2.2.2. Rice cultivation under salt treatment

2.2.2.1. Germination and sowing

Seeds of Aychade variety were surface sterilized in EtOH 95% (v/v) for 10 min., and washed repeatedly in distilled water. Seeds were placed on filter paper (Whatman No.1) and immersed in 5 mL reverse osmosis water in Ø 9 cm Petri dishes (20-25 seeds per dish) (a total of 30 Petri dishes). The Petri dishes were wrapped with parafilm to reduce evaporation. Afterwards they were placed in growth chamber at 28°C (14 h full light and 80% humidity). Germinated lasted for 4 days. After that homogeneous seedlings were transplanted into 330 pots filled with the soil and fertilizer mixture (2 seedlings per pot). They were grown under controlled conditions, a 12-h-photoperiod, 28/20°C day/night and relative humidity of 70 - 80%. Pots were irrigated every two days for one week with 100 mL of reverse osmosis water (RO water) until the second week after sowing.

2.2.2.2. Seedling stage

In the second week of growth homogeneous seedlings were selected in order to obtain 330 pots each containing one seedling. All of pots were placed in 4 trays. Sixty-six pots per tray (400 L, 100x200x20 cm³) were placed into tray 1, 2 and 3, and one hundred and twenty-two pots were placed in 600 L (100 x 300 x 20 cm³) of tray 4 (**Figure 18a**). The trays were filled with RO water (50 L per tray) up to 5 to 6 cm of the height of the pots until the BT stage (33 DAS).

2.2.2.3. Salinity stress treatment and water management:

Reverse osmosis (RO) water was used for control treatment (Electrical conductivity $EC_w < 500 \mu S.cm^{-1}$). Salt solution (30 mM NaCl) yielding an electrical conductivity $EC_w = 3800 \pm 400 \mu S.cm^{-1}$ was used for salinity conditions (**Table 8**). To introduce salt stress soil from pots were rinsed twice over two days with salt solution (300 mL of salt solution per pot each time). After that pots were kept in contact with saline water (NaCl 30 mM) (50 L per tray) introduced in the trays until the end of salt stress application. After salt stress treatment, soil from pot was irrigated with RO water four times over two days (300 mL of RO water per pot each time) to remove salt (called desalinized condition). Saline solution in the tray was then replaced by RO water.

Table 8. Management of salt treatment during rice plant cultivation.

Conditions	$EC_w (\mu S.cm^{-1})$	Water irrigation	NaCl (mM)
salt stressed	3800 ± 400	Salt solution	30
control	< 500	RO	0
desalinized	< 500	RO	0

There were five treatments, each comprised 66 pots which were salinized at different period during plant growth as following (**Figure 18b**).

5 Sequences of stress defined by the stage and the duration of the salt stress

- T0 = no salt treatment through cultivation as control
- T1 = salt treatment from BT until middle tillering (MT) as vegetative (I) phase for 14 days
- T2 = salt treatment from MT until panicle initiation (PI) as vegetative (II) phase for 14 days
- T3 = salt treatment from BT until PI as whole vegetative phase for 28 days
- T4 = salt treatment starting from PI until FLO as reproductive phase for 21 days

In greenhouse design, there are four trays for five treatments. Therefore plants from two treatments subjected to the same salinity treatment were placed together in the tray 4 due to its higher capacity **Figure 18a**. For example, plants from T1 and T3 treatments were salt treated similarly at vegetative (I), BT to MT stages as well as T2 and T3 treatments at vegetative (II), MT to PI as shown in **Figure 18b**.

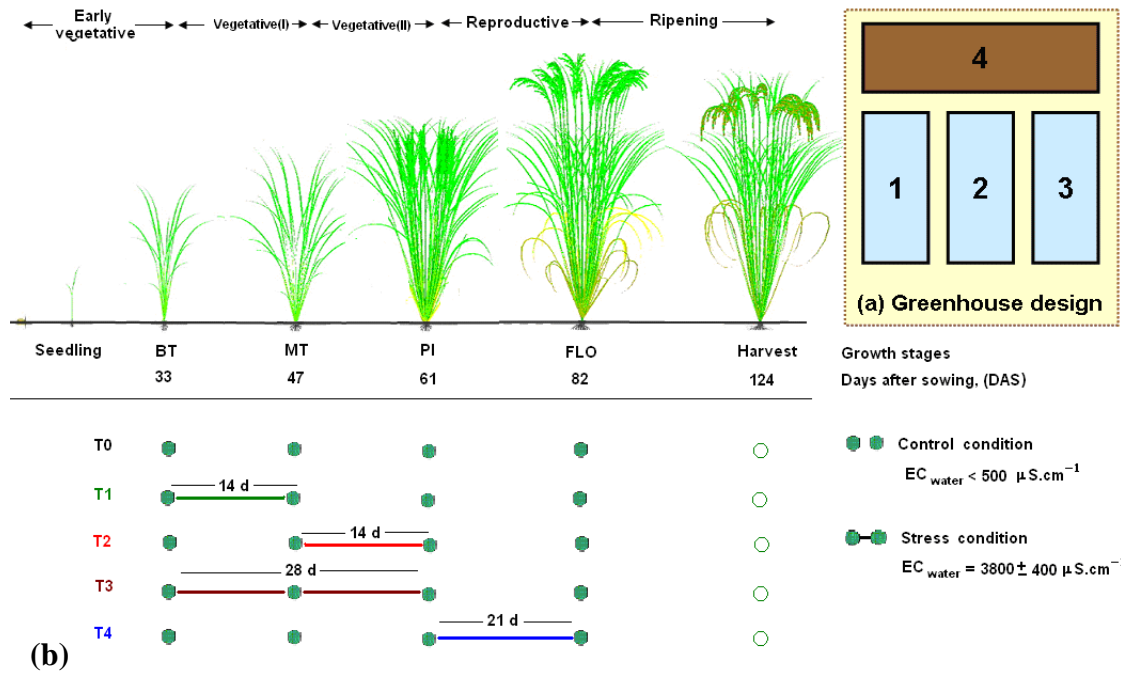


Figure 18. Experimental design of salt stress treatment during rice plant growth (b) and position of four trays in greenhouse, brown box for salt stress treatment and the others for no salt stress and desalinized conditions (a).

The level of irrigation water in trays was controlled (height of 5-6 cm) from 14 DAS up to 110 DAS and then stopped until harvesting. Electrical conductivity of saline solution in trays was regularly controlled at least twice a week by an electrical conductivity meter (Figure 19).

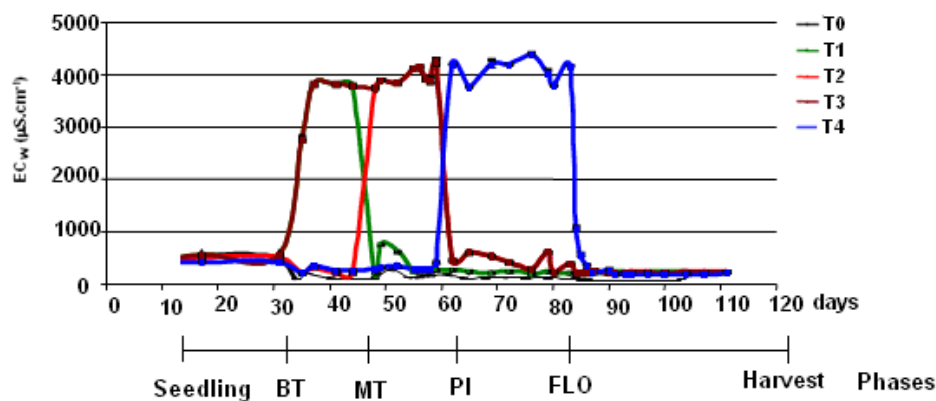


Figure 19. EC_w values ($\mu\text{S}\cdot\text{cm}^{-1}$) during rice plant cultivation. BT=beginning tillering stage, MT =middle tillering stage, PI = panicle initiation stage and FLO= flowering stage.

2.2.2.4. Sampling of rice plants

Sampling from rice plant was performed on four growth stages as shown in **Table 9**. Leaves were obtained from three stages (MT, PI and FLO stages), rice grains from harvest stage. Experimental setup comprised of 5 treatments (T0-T4). Each treatment for each stage had three plants replicates. At vegetative phase plants from MT and PI stages were harvested at 47 and 61 (DAS) respectively. 9 plants from each treatment, i.e., 45 plants for each stage were sampled. At reproductive phase (FLO stage), 12 plants from each treatment, i.e., 60 plants from this stage were harvested at 82 (DAS). At ripening phase, 18 plants from each treatment, i.e., 90 plants from this stage were harvested at 124 (DAS) to recover grains (**Table 9, Figure 20**).

Table 9. Number of sampling on each growth stage from five treatments

Stage	MT	IP	FLO	Harvest
(1) Harvesting (DAS ^a)	(47)	(61)	(82)	(124)
(2) Number of replicates per treatment	3	3	3	3
(3) Number of plants per replicate	3	3	4	6
(4) Number of plants per treatment (2)x(3)	9 ^c	9 ^c	12 ^c	18 ^c
(5) Number of plants per stage (4)x 5 ^b	45	45	60	90

Note: a= days after sowing, b = There were 5 treatments (T0-T4), c = number of plants per treatment were taken to analyze dry weight, 2AP, proline and GABA.

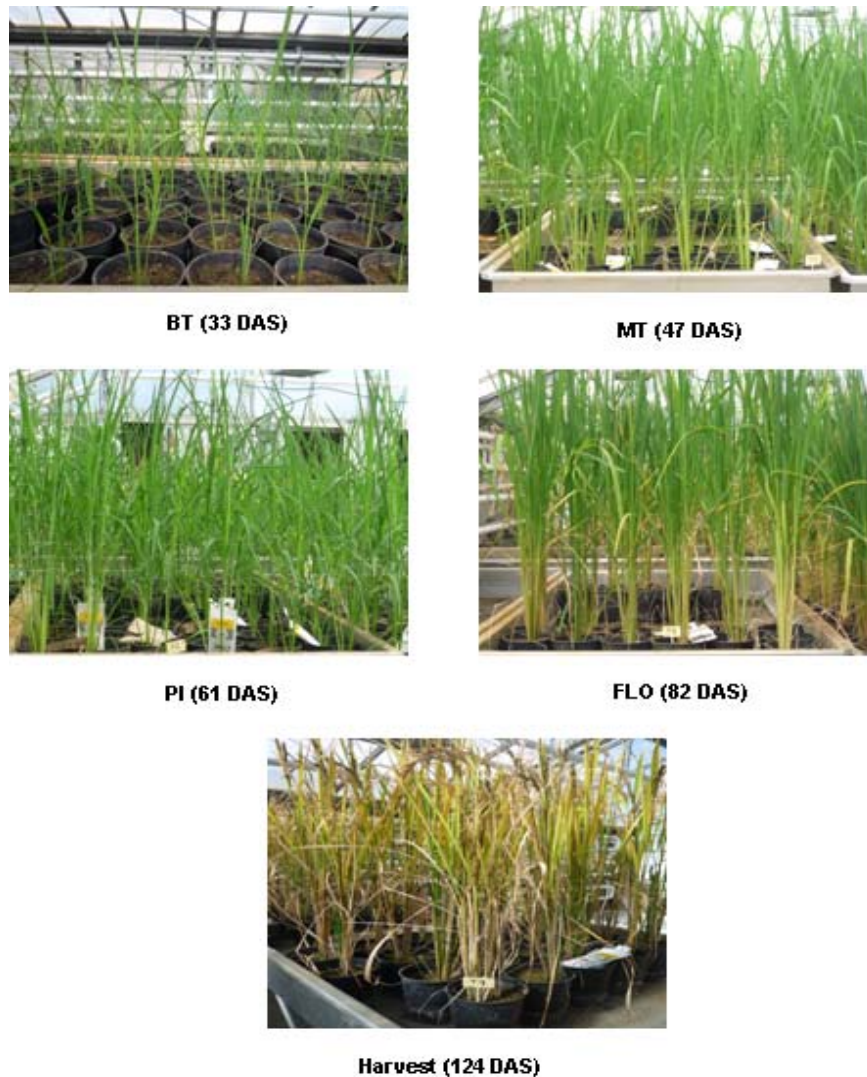


Figure 20. Development of rice plant in greenhouse

2.2.3. Measurements of other parameters

2.2.3.1. Morphological parameters and yield components

On each harvesting, plant development were measured on several parameters of morphological traits and yield components i.e., number of tillers, number of panicle, plant height and %dry weight of leaf and grain samples as shown in **Table 10**. On harvest stage, panicle length, thousand grain weight (TGW), dry weight of plant and panicle were also determined. Note that one of three plants replicates from each treatment was taken to measure growth parameters. The numbers of plants used for determination were 3 for MT and PI stages, 4 for FLO and 6 for harvest (**Table 10**). For % dry weight determination was done from three plants replicates for each treatment 9 to 18 plants according to each growth stage were sampled (**Table 9, 10**).

Table 10. List of morphological parameters and yield components were measured on each growth stage.

Measurement	MT	IP	FLO	Harvest
Harvesting (DAS) ^a	(47)	(61)	(82)	(124)
number of plant measured per treatment (from one plant replicate)	3	3	4	6
Plant height	√	√	√	√
number of tiller	√	√	√	x
number of panicle	x	x	√	√
Panicle length	x	x	x	√
^b TGW	x	x	x	√
^c %Dry weight (sample)	(leaves)	(leaves)	(leaves)	grains
Vegetative biomass	shoot	shoot	shoot	shoot, panicle

Note: √ = measured, x = no measured, a = days after sowing, b = thousand grain weight,
c = % dry weight was measured by three plants replicates per treatment.

2.2.3.2. Measurement of dry weight of rice plant and conservation of sample for analysis

Leaf samples (0.5 g), were cut into small pieces and placed on aluminium bowl. Similarly 10 grains were placed on aluminium bowl. Both materials were dried at 70°C in an oven to have a constant weight (3 days) to determine % dry weight. The rest of sample was immediately frozen in liquid nitrogen, and stored at – 20 °C until required for 2AP, proline and GABA analysis. Following all sampling for leaves and grains remaining plant (excluding roots) from pots was recovered, dried as mentioned above to determine dry mass.

2.3. 2-Acetyl-1-pyrroline (2AP) analysis

2.3.1. Synthesis of isotope-labeled 2-acetyl-1-pyrroline

2-Acetyl-1-pyrroline (2AP) and its deuterated analogue were synthesized at the Institut des Biomolécules Max Mousseron (IBMM) de l'Ecole Nationale Supérieure de Chimie (Montpellier, France).

Deuterate-2-acetyl-1-pyrroline (2AP-*d*2) (**2**) was synthesized as previously described (Maraval *et al.*, 2010). Briefly L-glutamic acid as a starting material was cyclised and followed by deacetylation of amide to amine. 5-acetylpyrrolidin-2-one (**1**), lactam compound, was obtained after purification by column chromatography on silica gel (**Figure 21**). 2AP-*d*2 (**2**) was then obtained in 3 steps; a protecting acetyl group with triethylorthoformate, followed with reduction by using LiAlD₄, oxidizing reagent as deuterium source and the deprotecting acetyl group together with oxidation reaction as the last step. 2AP-*d*2 (**2**) was extracted by diethyl ether and then identified by GC-MS. 2AP was synthesized in similar way to 2AP-*d*2 using LiAlH₄, oxidizing reagent instead of LiAlD₄.

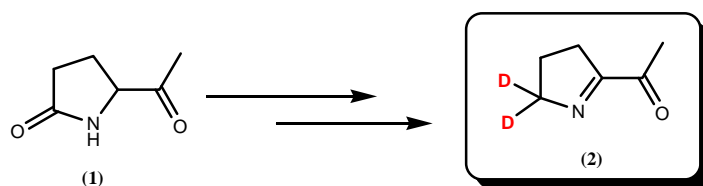


Figure 21. Schematic representation of 2AP-*d*2 synthesis by 5-acetylpyrrolidin-2-one as intermediate

Diethyl ether solutions of 2AP-*d*2 and 2AP were taken in ethanol for further analysis by SPME. For this, 2AP-*d*2 and 2AP solutions were individually and gently evaporated under nitrogen gas and concentrated approximately to 100 µL. Absolute ethanol was then added to make a final volume of 500 µL as stock solution. Concentration of analytes was determined by GC-MS-EI analysis using collidine (2,4,6-trimethylpyridine) as an internal standard (*see* 2.3.5). 2AP and 2AP-*d*2 solutions were used to make the calibration curves for 2AP quantification in rice samples (leaves, grains) (*see* 2.3.5). All stock solutions were stored at -80°C prior to use.

2.3.2. 2AP quantification

For all rice samples, seedlings, leaves and grains the concentration of 2AP was determined by stable isotope dilution assay (SIDA) through solid-phase microextraction (SPME), gas chromatography-positive chemical ionization-tandem mass spectrometry (GC-PCI-MS/MS) (Maraval *et al.*, 2010)

2.3.3. SPME analysis

Stable-Flex divinylbenzene/Carboxen/ polydimethylsiloxane50/30 μ m fiber (Supelco, Bellefonte, PA, USA) was used for Solid Phase Microextraction (SPME). Note that before the first use, it has to be conditioned at 270°C for 1 h according to the supplier's recommendation.

Leaf or grain samples were powdered in the presence of liquid nitrogen by a mortar (for leaf samples) and by a grinder machine (SEB coffee grinder model Prep Line 850, France) (for grain samples). All of powdered samples were stored at -20°C until analyzed.

0.5 g of powdered sample (shoot, leaf or grain) with 4 mL of sodium carbonate buffer pH 9.2 (0.1 M) were placed subsequently in a 10 mL glass vial containing a magnetic bar covered with glass. 8 μ L of 2AP-*d*2 stock dilution (24.42 μ g.mL⁻¹ in ethanol) as an internal standard solution was added to the mixture. The vial of sample was kept in an ice bath to avoid possible loss of the analytes during preparing steps. The vial was then sealed with a PTFE septum and magnetically agitated (800 rpm) at 80°C. After 5 min of equilibration, the SPME fiber was subjected to the headspace for 20 min at 1 cm over the sample surface (**Figure 22**) (Maraval *et al.*, 2010) Each sample was independently extracted in duplicate run.

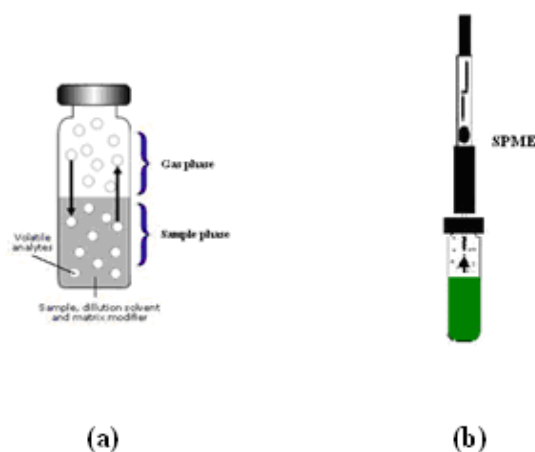


Figure 22. Equilibrium phase (a) and headspace SPME-sampling (b)

2.3.4. GC-MS analysis

Following SPME, the analytes from fiber were desorbed in the injector port (5 min., splitless mode) of CP-3800 gas chromatograph, coupled with a Saturn 2000 ion-trap mass spectrometer (Varian, Walnut Creek, CA, USA). Chromatographic separation was accomplished with a DB-Wax (J&W Scientific, Folsom, CA) fused silica capillary column (30 m x 0.32 mm i.d., film thickness = 0.5 μ m). Helium gas was used as the carrier gas in flow rate 1 mL.min⁻¹. The injection port temperature was 250°C. The temperature of GC column was 50°C for 1 min, then at 2°C.min⁻¹ to 85°C, followed at 30°C.min⁻¹ to 245°C and held for 8 min at 245°C. Ion trap temperature of MS was 150°C, manifold and transfer line temperatures were 45 and 250°C, respectively. The mass range scanned was from m/z 60 to 120. Two modes of ionisation were preformed. Electronic impact mode (MS/EI) was generated at 70 eV. For positive chemical ionisation (PCI) acetonitrile was used as reagent gas. MS-MS conditions were determined through Multiple Reaction Monitoring (MRM) mode by injection of solutions of 2AP and 2AP-*d*2 (**Table 11**).

Quantification of 2AP in rice samples (shoot seedling, leaves and grains) were performed by GC-PCI-MS-MS technique. 2AP-*d*2 was used as an internal standard. The mass spectra were obtained by scanning from m/z 66 to 120 with positive chemical ionization (acetonitrile as reagent gas) tandem mass (PCI-MS/MS) mode. The optimum voltages for non resonant excitation collision-induced dissociation (CID) are 47.5 and 47.0 V for 2AP and its deuterated analogue, respectively (**Table 11**).

Table 11. Preparation ion in Multiple Reaction Monitoring (MRM) mode by positive chemical ionization tandem mass (PCI-MS/MS) analysis.

Analyte	Parent Ion Mass	Daughter ions Mass	Isolation Window	Waveform Type	Excitation Storage Level	Excitation Amplitude
2AP	112.0	70.0	1.0	Non-resonant	48.0	47.5
2AP- <i>d</i> 2	114.0	72.0	1.0	Non-resonant	48.0	47.0

Under conditions of GC-PCI-MS-MS technique, the retention times of 2AP and its deuterated analogue were found at 11.65 and 11.57 min, respectively. Meanwhile molecular ion of 2AP and its deuterated analogue were observed at m/z 112 and 114 respectively. Both of parent ions were further fragmented to give the daughter ions of 2AP and 2AP-*d*2, m/z 70

and 72 respectively. Quantification of 2AP was performed through calibration curve of leaves or grains matrix plotted separately. The calibration curve was plotted between the area ratios of the daughter ions of 2AP and 2AP-*d*2 as 70 and 72 respectively versus their quantities ratio (Maraval *et al.*, 2010). The results are the average of the analysis of duplicate extractions. The coefficient of variation of 2AP analysis was less than 10%. The results were expressed as $\mu\text{g.kg}^{-1}$ (mean \pm SD) on dry weight basis.

2.3.5. Construction of calibration curve

To avoid matrix effect during SMPE technique, two calibration curves were prepared using leaves (0.5 g) or grains (0.5 g) from Gladio x Fidji-K2, a non fragrant rice variety. The amounts of 2AP and 2AP-*d*2 were determined differently in the case of leaves and grains. In the leaves matrix a range of 0.006 - 3.757 μg of 2AP while in the grain matrix a range of 0.006 - 1.503 μg of 2AP was used. Note that each time a constant amount of 2AP-*d*2 (0.195 μg) were introduced together in the matrix medium as internal standard.

Calibration curve was prepared in duplicate for each matrix. Both calibrations showed a good linearity with regression coefficients of 0.9961 and 0.9974 for leaves and grain matrix, respectively (Figure 23 and 24).

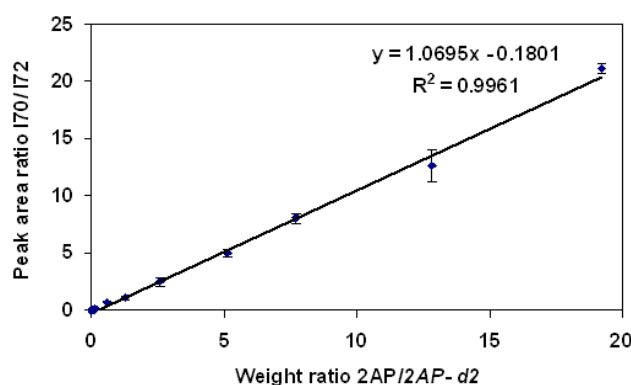


Figure 23. Calibration curves for 2AP in leaves matrix.

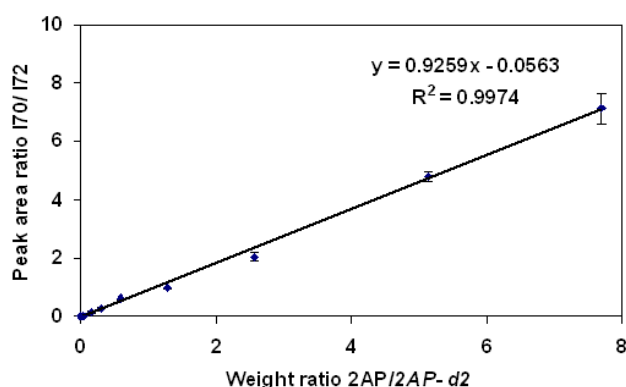


Figure 24. Calibration curves for 2AP in grain matrix

2.4. Proline and GABA analysis

Proline and GABA concentrations in rice samples were determined through 4-(dimethylamino) azobenzene-4'-sulfonyl chloride (dabsyl) derivatives.

2.4.1. Extraction proline and GABA from rice sample

Proline and GABA extraction was performed as described by Gay *et al.* (2010). 100 mg of leaf or grain powder samples taken in 10 mL of tube was added with 4 mL sodium carbonate buffer (pH 9.0, 0.1 M) and 50 μ L of 25 μ mole.mL⁻¹ carboxymethylcysteine (CMC) solution as internal standard. The tube was covered with fitting cap at room temperature and then undergo to gentle rotation of 8-10 rpm (Agitest 34050, Bioblock Scientific, Illkirch, France) for 1 h. Following filtration on 0.45 μ m Millipore filter, 25 μ L of the extract was mixed with 75 μ L of sodium carbonate buffer (pH 9.0, 0.1 M) and 100 μ L of 6mM dabsyl (4-(dimethylamino) azobenzene-4'-sulfonyl chloride) solution in acetonitrile. After incubation for 10 min at 70°C, the solution was added with 300 μ L phosphate buffer (pH 7.0, 9 mM). 20 μ L of the medium was analyzed by a reverse phase (C 18) High Liquid Chromatography Performance (HPLC) technique.

2.4.2. Conditions of HPLC analysis

HPLC was Dionex liquid chromatograph equipped with model P680 pumps, an ASI 100 autosampler, a UVD 340U diode array detector coupled to a HP ChemStation (Dionex, France). The column was a reverse phase (C 18) column, 250 mm \times 4.6 mm (i.d.); 5 μ m (Symmetry, Waters). Mobile phase A consisted of 1% (v/v) 0.9 M NaH₂PO₄, 4% (v/v) dimethyl formamide, and 0.1% triethylamine in water. The pH was adjusted to 7.55. Mobile phase B was acetonitrile-water (80:20, v/v). The elution was performed at 1 mL.min⁻¹ with a linear gradient from 28% B at 0 min to 42% B at 30min. The column was then rinsed with 100% B then equilibrated with 28% B for at least 10 min.

The concentration of proline and GABA in the samples were determined by using standard curve. Each sample was analyzed in duplicate. The coefficient of variation of proline and GABA analysis was less than 10%. The levels of targeted compounds were expressed as μ g.g⁻¹ DW (mean \pm SD).

2.5. Statistical analysis

Analysis of variance (ANOVA) with mix model test, regression analysis and multiple comparisons of means at $p < 0.05$, Principal Component Analysis (PCA) were performed with the XLSTAT Pro 2010 software (Addinsoft, France).

Part 1

Results and Discussion

1. PUBLICATION No. 1

Salt stress during rice seedling culture induces 2-acetyl-1-pyrroline biosynthesis, a dominant flavor compound in fragrant rice

Submission in November 2011 in *Journal of “Industrial crops and products”*

**Janchai POONLAPHDECHA^{a,b}, Pascal GANTET^{c,d}, Isabelle BOURRIE^c,
Isabelle MARAVAL^a, Renaud BOULANGER^b, Ziya GUNATA^{a*}**

^a UMR Qualisud, Université de Montpellier II, place E. Bataillon, 34095 Montpellier Cedex 5, France

^b UMR Qualisud, CIRAD, 73 Rue J.F. Breton, 34398 Montpellier Cedex 5, France

^c Université Montpellier 2, UMR DAP, Bat 15, CC 002, Place Eugène Bataillon, 34095 Montpellier Cedex 5, France

^d University of Science and Technology of Hanoi, Department of Biotechnology and Pharmacology, Institute of Agricultural Genetics, Laboratoire Mixte International Rice Functional Genomics and Plant Biotechnology, Hanoi, Vietnam

*Corresponding author. Tel. (33) 4 67 14 93 21 - Fax (33) 4 67 14 42 92

e-mail : zgunata@univ-montp2.fr

2. PUBLICATION No. 2

Effect of timing and duration of salt treatment during growth of a fragrant rice variety on 2-acetyl-1-pyrroline biosynthesis, key flavour compound in rice

Submission in December 2011 to “*Journal of Agricultural and Food Chemistry*”

**Janchai POONLAPHDECHA^{a,b}, Isabelle MARAVAL^a ; Sandrine ROQUES^c,
Alain AUDEBERT^c, Renaud BOULANGER^b, Xavier BRY^d, Ziya GUNATA^{a*}**

^a UMR Qualisud, Université de Montpellier II, place E. Bataillon, 34095 Montpellier Cedex 5, France

^b UMR Qualisud, CIRAD, 73 Rue J.F. Breton, 34398 Montpellier Cedex 5, France

^c UMR AGAP, CIRAD, Avenue Agropolis, 34398 Montpellier Cedex 5, France

^d 13M, Université de Montpellier II, place E. Bataillon, 34095 Montpellier Cedex 5, France

*Corresponding author. Tel. (33) 4 67 14 93 21 - Fax (33) 4 67 14 42 92

e-mail : zgunata@univ-montp2.fr

Part 1



Conclusions

Effect of salinity on the biosynthesis of 2-acetyl-1-pyrroline (2AP), proline and GABA was studied by conducting the experiments with seedlings in a liquid medium and in the pots in a greenhouse. Aychade a fragrant rice variety was used in both experiments.

Seedling growth medium was added with four levels of salt solution (1.7, 17, 85 and 170 mM NaCl). At these concentrations of NaCl seedling growth was not affected clearly. However osmotic stress may have been occurred at 85 and 170 mM. Indeed significantly higher levels of proline, a osmolyte compatible compound were detected at 85 and 170 mM of NaCl. Interestingly the concentration of 2AP increased significantly in the seedlings grown in the presence of salt whatever salt concentration in the medium. Moreover this increase was correlated positively with the concentration of the salt in the medium. Proline synthesis may not be the limiting factor of 2AP biosynthesis in moderate salt stress (1.7 to 17 mM NaCl) since proline level at these salt concentrations was not different from that of control. In contrast to 2AP and proline, the concentration of GABA was only affected at 170 mM of NaCl leading to a decrease. At strong stress condition, conversion of γ -amino butyraldehyde (GABald) into GABA by *BADH2* (Bradbury *et al.*, 2008) may have been limited. The decrease in GABA level at the highest salt concentration may be explained by a higher yield of transformation of GABald to 2AP rather than to GABA.

In greenhouse during different plant growth stages only one level of salt solution (30 mM) giving an electrical conductivity of $3800 \pm 400 \mu\text{S}.\text{cm}^{-1}$ was applied to induce osmotic stress. Timing and duration of salt solution differed according to growth stages under four treatments (T1-T4). Reverse osmosis water ($\text{EC}_{\text{water}} < 500 \mu\text{S}.\text{cm}^{-1}$) was used as control (T0). In general salt treatment did not affect strongly plant growth and yield components. 2AP biosynthesis in leaves and grains was influenced by growth stages and salinity treatment. The highest concentrations of 2AP in leaves were observed in plants salt-treated during either first vegetative phase (T1) or whole vegetative phase (T3). In contrast, 2AP synthesis in the leaves from second vegetative phase (T2) and from reproductive phase (T4) was not in general significantly different from the control. Interestingly salinity treatments resulted in a significant increase in 2AP amount in grains whenever salt solution was applied. In particular the salinity treatment during whole vegetative and reproductive period exhibited the highest concentration of 2AP in grains. However the latter caused a significant decrease in grain yield.

A significant positive relationship between 2AP and proline levels were observed in the leaves. This suggests that proline may have been used for the biosynthesis of 2AP in accordance with a previous work on rice seedlings and callus (Yoshihashi *et al.*, 2002b). On the contrary there was no correlation with 2AP and proline levels in the grains. Here two possibilities of accumulation of 2AP in grains were considered: firstly the transport of 2AP from the leaves into grains. This hypothesis has been supported by the fact that proline level was quite similar in the grains from the control and from salt-treated plants. Secondly proline translocated from the leaves to the grain may have participated to 2AP synthesis in the grain.

Finally the concentrations of GABA were not correlated with 2AP synthesis as well as salinity treatment. However high concentrations of GABA were observed in the leaves from the plants salt-treated during vegetative phase, suggesting its participation to mechanism of osmoprotection.

Part 2

*Biosynthesis of 2AP:
role of 1-pyrroline and
source of acetyl group donor*

2-Acetyl-1-pyrroline (2AP) is major flavoring compound of fragrant rice varieties imparting pop-corn like aroma (Buttery *et al.*, 1982; Buttery *et al.*, 1983a). Increases in 2AP level were observed through feeding of rice seedlings and calli with amino acids such as proline, ornithine and glutamic acid (Yoshihashi *et al.*, 2002b). Proline was found to be amino acid leading to the highest increase in 2AP level. Microbial production of 2AP from amino acids has also been demonstrated. *Bacillus cereus* (Adams and De Kimpe, 2007; Romanczyk *et al.*, 1995) was able to use proline and ornithine as amino acid precursor (Romanczyk *et al.*, 1995) while *Lactobacillus hilgardii* ornithine (Costello *et al.*, 2001; Costello and Henschke, 2002). Recent studies proposed two pathways for 2AP biosynthesis in rice via proline (Bradbury *et al.*, 2008) and glutamic acid (Huang *et al.*, 2008) but both through 1-pyrroline. The latter was also found an important intermediate in the formation of 2AP by Maillard reaction (Hofmann and Schieberle, 1998). However nothing is demonstrated yet on the role of 1-pyrroline in 2AP biosynthesis in rice. In addition the last step of 2AP consisting of the acetylation of 1-pyrroline ring has been subject of only one recent paper (Huang *et al.*, 2008). Methylglyoxal was proposed as acetyl donor on the basis of its abundance in fragrant rice varieties and its ability to react with pyrroline-5-carboxylic acid.

In this chapter role of 1-pyrroline in the biosynthesis of 2AP not only in fragrant but also in non fragrant rice varieties was studied through feeding of rice callus with this cyclic imine. To investigate the origin of acetyl group rice calli were generated in agar medium supplemented with putative labeled precursors, D-glucose ($^{13}\text{C}_6$), sodium acetate (1,2- $^{13}\text{C}_2$) and sodium octanoate (1,2,3,4- $^{13}\text{C}_4$). Incorporation of labeled C13 atoms into 2AP was studied by electronic impact (EI), positive chemical ionization and tandem MS-MS mode.

A project of publication was prepared to be submitted in few months entitled as follows:

“Some clues on 2-acetyl-1-pyrroline biosynthesis, a major flavoring compound in fragrant rice: key role of 1-pyrroline and source of acetyl group”

Part 2

Materials and Methods

Experiment 3: Effect of 1-pyrroline on the biosynthesis of 2AP in rice

1.1. Plant material

In the experiment, fragrant and non fragrant rice, Aychade and Gladio x Fidji K2 varieties, respectively were tested. They were obtained from Camargue field (2009) in France.

1.2. Synthesis of 1-pyrroline and quantification

1-Pyrroline was synthesized by acid hydrolysis of 4-aminobutyaldehyde diethyl acetal. This method was modified from Struve and Christophersen (2003) (**Figure 25**). Starting with 6 mmol (0.97g) of 4-aminobutyaldehyde diethyl acetal (Sigma Co., > 90%) in a 50-mL pear-shaped flask containing a magnetic stir bar, cooled in an ice-water bath to 0°C, and then added 24 mmol (12 mL) of ice cold 2 M HCl. The reaction mixture was magnetically stirred (800 rpm) for 25 min in an ice bath before gently adding 48 mmol (18 mL) of 2.67 M K₂CO₃ solution and then stirred continuously. After 5 min, reaction solution was measured (pH > 12). The cool alkaline solution was allowed to return to room temperature. 1-pyrroline in final solution was analyzed by TLC using silica gel as stationary phase and methanol:acetone:concentrated HCl (90:10:4, v/v) as eluant and detected with 4% ninhydrin in alcohol.

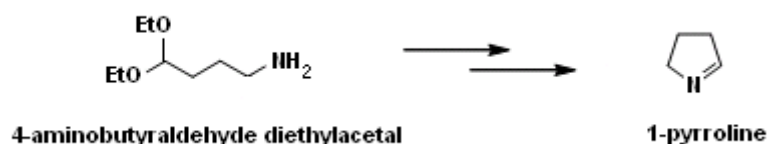


Figure 25. Schematic representation of 1-pyrroline synthesis

1-Pyrroline from reaction mixture was recovered by distillation under vacuum and trapping with liquid nitrogen using Solvent Assisted Flavor Extraction (SAFE) apparatus (**Figure 26**). The assembly used was similar to that described by Engel *et al.*, (1999) as a fast and careful method for the isolation of volatiles. The reaction mixture (30 mL) was poured in the distillation flask maintained at 30°C in a water bath (2). Vacuum was 10 mbar and the volatiles were rectified in the distillation head (3). The distillate was trapped in a flask cooled with liquid nitrogen (4). The drawer flask (5) contained also liquid nitrogen to avoid loss of volatiles. Distillation lasted for 1 h. The distillate was then extracted with CH₂Cl₂ (3 x 5 mL). The combined organic extract was dried with anhydrous sodium sulphate and evaporated gently using nitrogen gas approximately to 100 µL. This volume was then made to a final

volume of 500 μL with absolute ethanol. The stock solution was stored at -20°C . It was used within three days since instable (Robacker *et al.*, 1997).

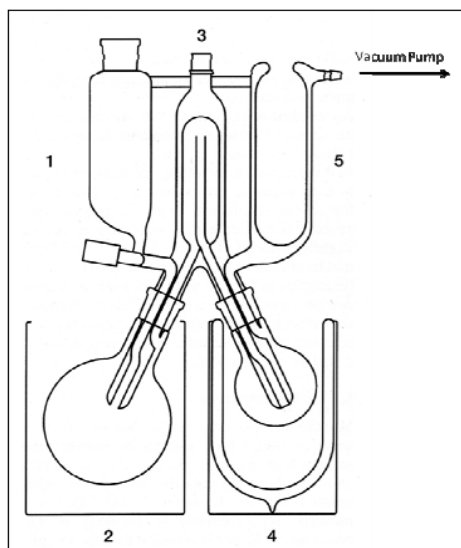


Figure 26: SAFE apparatus for the distillation of 1-pyrroline synthetic solution (Belitz, *et al.*, 2004)

1-Pyrroline was quantified from stock solution by GC-MS analysis in PCI mode. Pyrrolidine (Aldrich, 99%) (9.875 mg.mL^{-1} in ethanol) was used as an internal standard. Each quantification was done in duplicate. The mixture of 1-pyrroline and pyrrolidine solution was subjected to GC-MS analysis on CP-3800 gas chromatograph coupled with a Varian Saturn 2000 ion-trap mass spectrometer (Varian, Walnut Creek, CA, USA). Chromatographic separation was performed on a RTX-35 amine (Restek Corp., Bellefonte PA, USA) fused silica capillary column ($30 \text{ m} \times 0.25 \text{ mm ID}$, film thickness $1 \mu\text{m}$), a special GC column for separation of basic compounds. GC conditions were as follows: split injection mode (1:10) at 200°C ; initial oven temperature 30°C (held for 1 min), followed at $2^{\circ}\text{C.min}^{-1}$ to 55°C , then at $8^{\circ}\text{C.min}^{-1}$ to 200°C and held for 5 min at 200°C . Helium was used as carrier gas (1 mL.min^{-1}). The mass range scanned was m/z 29 to 350. Chemical ionisation (CI) reagent gas was acetonitrile. The mass spectra were obtained by scanning from m/z 35 to 130. Ion trap temperature was 150°C , manifold and transfer line temperatures 45 and 250°C respectively. 1-Pyrroline and pyrrolidine were eluted at 9.68 and 9.08 min. Pseudo-molecular ions were 70 and 72 for 1-pyrroline and pyrrolidine respectively.

1.3. Rice callus generation

1.3.1. Composition of medium for rice callus culture

The composition of medium so-called NB medium for rice callus culture is given in **Table 12** (Gamborg et al., 1968). The medium mainly consists of N6 macro elements, B5 micro elements (Chu, 1975), 500 mg.L⁻¹ proline, 500 mg.L⁻¹ glutamine, 2.5 mg.L⁻¹ 2,4-D and 3.0% sucrose (Duchefa biochemie, Netherlands). pH of medium was adjusted with 1 N NaOH to 5.8. and then the medium was added with agar (0.26%; w/v). Sterilization was done at 121°C for 20 min. Agar NB medium was prepared in Ø 9 cm Petri dishes. The dishes were wrapped with Parafilm to protect microbial contamination.

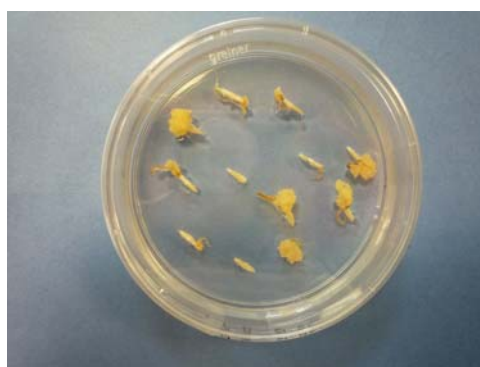
1.3.2. Rice callus induction

Paddy seeds (*O. sativa* L.) of Aychade, a fragrant rice variety and Gladio x Fidji K2, a non fragrant rice variety were used in this experiment. Dehusked seeds were surface-sterilized sterilized in 70% (v/v) ethanol for 3 min following by shaking vigorously every 5 min for 50 min in a sodium hypochlorite solution (40%; v/v). The seeds were then rinsed four times with sterilized distilled water. The seeds were then blotted on sterile Whatman filter paper sheets after the last rinsing with sterile distilled water. After that the sterilized seeds were placed on NB agar medium in Petri dishes. Rice seeds were grown in the dark chamber at 28°C and 60-70% humidity. After six weeks, fresh calli as shown in **Figure 27** were recovered from seeds and root for further analysis.

Table 12. The composition of NB medium used for rice callus culture.(Chu, 1978 ; Gamborg *et al.*, 1968)

Component	Concentration		Component	Concentration
Sucrose	30	g/L	N6 Macro 20x	(g/L)
Myo-inositol	100	mg/L	KNO ₃	56.6
L-Proline	500	mg/L	(NH ₄) ₂ SO ₄	9.26
L-Glutamine	500	mg/L	KH ₂ PO ₄	8.00
Casine hydrolysate	300	mg/L	MgSO ₄ .7H ₂ O	3.70
N6 Macro	50	mL/L	CaCl ₂ .2H ₂ O	3.32
B5 Micro	10	mL/L	B5 Micro 1000x	(mg/L)
FeNaEDTA	10	mL/L	MnSO ₄ .4H ₂ O	758
B5 Vitamines	10	mL/L	H ₃ BO ₃	300
^a 2,4 D solution (1mg/mL)	2.5	mL/L	ZnSO ₄ .7H ₂ O	200
			KI	75
			Na ₂ MoO ₄ .2H ₂ O	25
			CuSO ₄ .5H ₂ O	2.5
			CoCl ₂ .6H ₂ O	2.5
			FeNaEDTA 100x	g/L
			FeSO ₄ .7H ₂ O	2.78
			Na ₂ EDTA	3.72
			B5 Vitamins 100x	(mg/L)
			Nicotinic acid	100
			Pyridoxine-HCl	100
			Thiamine	1000

a = 2,4-Dichlorophenoxyacetic acid (2,4-D)

**Figure 27.** Rice callus induction of Aychade rice variety

1.4. Incubation of rice callus with putative precursors

Fresh rice calli (0.5 g.) were immersed in a test tube containing 4 mL of different media i.e., sodium phosphate buffer (0.1 M) at pH 5.8, pH 8.0 or Linsmaier and Skoog (LS) medium (pH 5.5) (Duchefa biochemie, Netherlands). Different levels of 1-pyrroline (from 1000 ppm to 7000 ppm) or L-proline (500 ppm) was added subsequently in the reaction medium. Control was composed of rice calli and medium. For blank tests, rice calli (0.5 g) were heated in the medium above (4 mL) at 98°C for 30 min. After cooling the medium, the heated calli were recovered and added with culture medium and putative precursors as mentioned above. The tubes were closed with a screw cap and incubated at 28°C in darkness for 12 h. Following incubation, the liquid medium was removed, calli were rinsed with distilled water and recovered for 2AP analysis.

Four experiments were conducted in relation with the nature of precursors and culture medium.

1.4.1. Effect of proline

In this experiment 0.5 g of rice calli from Aychade variety were immersed in a test tube containing 4 mL of LS medium (Duchefa biochemie, Netherlands) (pH 5.7) and L-proline (500 or 2000 ppm) (**Figure 28**). Control was the rice callus plus LS medium. Each treatment was tested in duplicate.

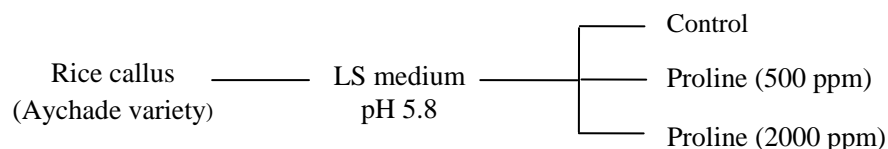


Figure 28. Study of effect of L-proline on the biosynthesis of 2AP in rice calli

1.4.2. Effect of pH

0.5 g of rice calli from Aychade variety were immersed in a test tube containing 4 mL of sodium phosphate buffer (0.1 M) at pH 5.8 or 8.0, plus 1-pyrroline (1000 ppm) (**Figure 29**). Control was composed of rice calli and phosphate buffer (0.1 M) at pH 5.8 or 8.0. The blank tests were performed with heated calli (*see 1.4*). Each treatment was tested in duplicate.

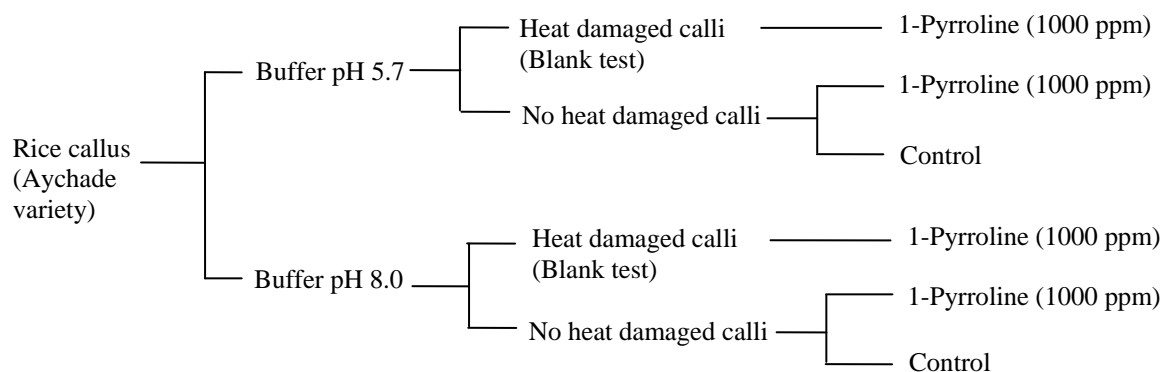


Figure 29. Study of effect of pH medium on the biosynthesis of 2AP in rice calli

1.4.3. Effect of 1-pyrroline

0.5 g of rice calli from Aychade (fragrant rice) and from Gladio x Fidji-K2 (non fragrant rice variety) were immersed independently in a test tube containing 4 mL of sodium phosphate buffer (0.1 M) at pH 8.0 and plus 1-pyrroline (1000 ppm) (**Figure 30**). Control composed of rice calli and phosphate buffer (0.1 M) at pH 8.0. Blank tests were done for each rice variety as described above (*see 1.4*). Each treatment was tested in duplicate.

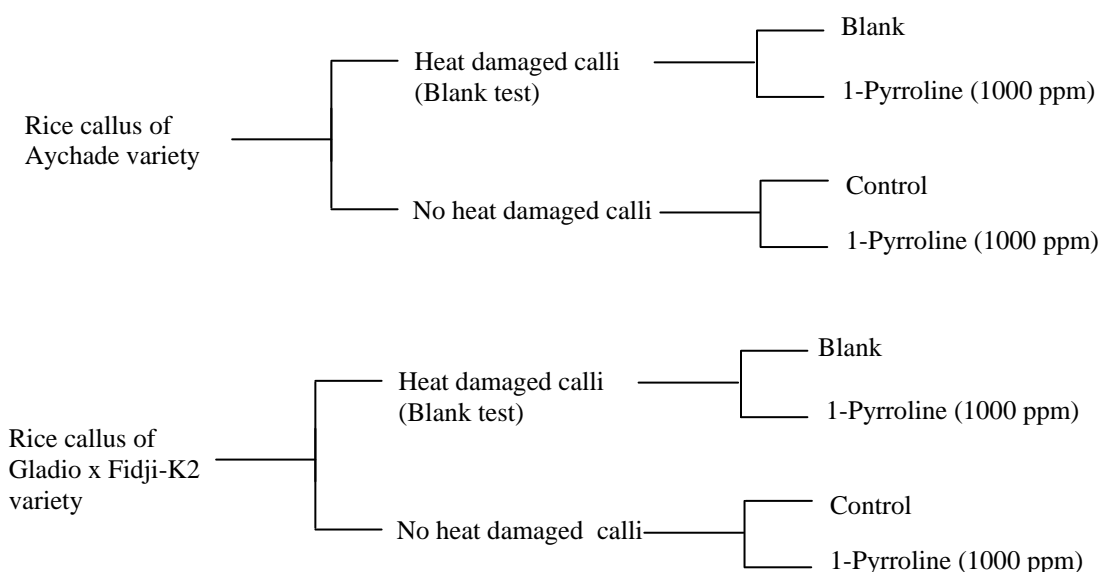


Figure 30. Study of effect of 1-pyrroline

1.4.4. Effect of levels of 1-pyrroline

0.5 g of rice calli, Aychade were immersed independently in a test tube containing 4 mL of sodium phosphate buffer (0.1 M) at pH 8.0 with different levels of 1-pyrroline (from 1000 ppm to 7000 ppm) **Figure 31**. Control was composed of rice calli and phosphate buffer. There were five blank tests (*see 1.4*) according to levels of 1-pyrroline used in non heat damaged calli. All of them were done in duplicate.

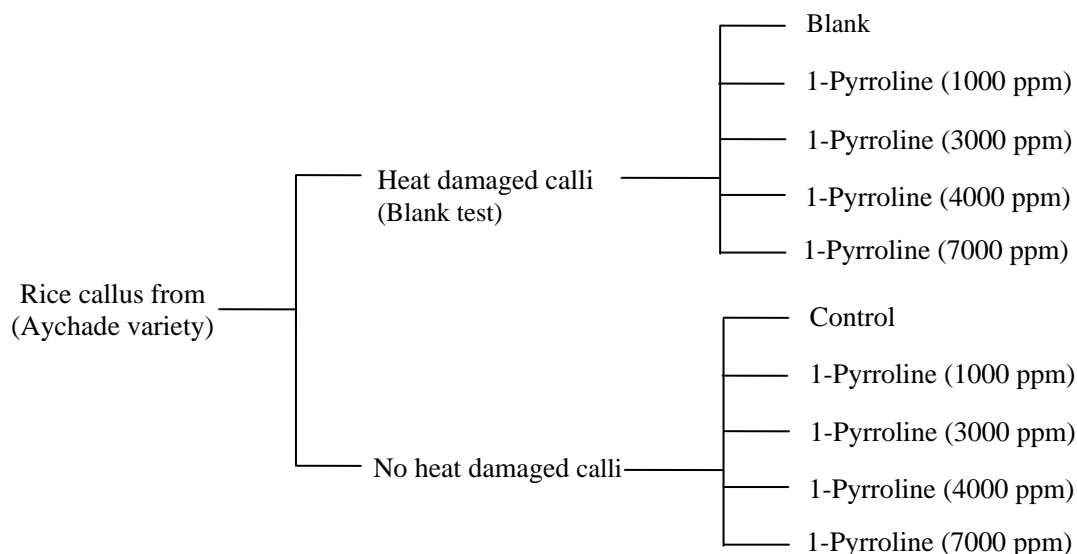


Figure 31. Study of the effect of levels of 1-pyrroline on the biosynthesis of 2AP in rice calli

1.5. Extraction of 2AP from rice calli

After incubation, 0.5 g of rice calli was finely ground under liquid nitrogen by a mortar. The powder was introduced into the vial (10 mL), added with 4 mL of sodium carbonate buffer (pH 9.2, 0.1 M) and 3 μL of 2AP-*d*2 solution ($38.68 \mu\text{g}\cdot\text{mL}^{-1}$ in ethanol) as internal standard. The vials were kept in an ice bath to conserve possible loss of the analytes during sample preparation step. They were covered with PTFE-faced silicone septum, and then heated at 80°C for 5 min under magnetical agitated using a glass magnetic stir bar (800 rpm). After equilibrium a Solid-phase microextraction (SPME) fiber (Stable-Flex divinylbenzene/Carboxen/ polydimethylsiloxane, DVB/CARIPDMS; 50/30 mm; Supelco, Bellefonte, PA, USA) was placed to the headspace for 20 min at 1 cm over the sample surface (Maraval *et al.*, 2010). Following extraction GC-MS analysis was performed. Each sample was extracted in duplicate. Note that the fiber was conditioned at 270°C for 1 hour before the first using according to the supplier's recommendation. Each sample was extracted in duplicate. Following extraction GC-MS analysis was performed.

1.6. GC-MS/MS analysis

2AP was analyzed by GC-MS using on a CP-3800 gas chromatograph, coupled with a Saturn 2000 ion-trap mass spectrometer (Varian, Walnut Creek, CA, USA). The injection port temperature was mounted with a 0.5 mm I.D. liner and maintained at 250°C. Desorption of analytes from SPME fiber was in splitless mode (1 min.). Chromatographic separation was accomplished with a DB-Wax (J&W Scientific, FoIson, CA) fused silica capillary column (30 m x 0.32 mm i.d., film thickness = 0.5 µm). Helium gas was used as the carrier gas in flow rate of 1 mL.min⁻¹. The temperature of GC oven was programmed to start at 50°C for 1 min, then the temperature was increased at 2°C min⁻¹ to 85°C and hence at 30°C min⁻¹ to 245°C and held for 8 min at 245°C .

For quantification of 2AP from the samples incubated with non labeled precursors (proline and 1-pyrroline), a stable isotope dilution assay involving SPME-PCI-MS/MS was used (Maraval *et al.*, 2010). The mass spectra were obtained by scanning from m/z 66 to 120. 2AP was quantified through area ratio between major daughter ions at m/z 70 and 72, from 2AP and of 2AP-*d*2 respectively (*see 2.3.4. in Experiment 2*). 2AP concentration was measured in duplicate analysis and was expressed as µg.g⁻¹ (mean ± SD).

1.7. Statistical analysis

Each experiment was performed in duplicate. The data were subjected to analysis of variance (ANOVA) at $p < 0.05$ with the XLSTAT Pro 2010 software (Addinsoft, France).

Experiment 4: Origin of acetyl group on the biosynthesis of 2AP**2.1. Plant material**

In the experiment, seeds of Aychade, a fragrant rice variety from Camargue field (2009) in France was used.

2.2. Rice callus generation**2.2.1. Composition of medium with labeled precursors**

Sucrose in NB medium (**Table 14**) used for rice callus culture was replaced by non labelled glucose and [U- ^{13}C]6-glucose (Isotec (Sigma Aldrich) Their concentration was 1.5 g/100 mL of medium. We checked that there was no change in the development of callus because of this modification. To avoid Maillard reaction during sterilization of the medium glucose and labeled glucose solution was filtered on sterile 0.5 μm filter and then introduced into NB medium previously sterilized. Other ^{13}C -labelled compounds, i.e., sodium acetate (1,2- $^{13}\text{C}_2$) and sodium octanoate (1,2,3,4- $^{13}\text{C}_4$) (Cambridge Isotope Laboratories, Andover, MA) were introduced into NB medium at 0.2 and 0.1 g/1L, respectively prior to sterilization. pH of medium was adjusted with 1 N NaOH to 5.8 and then agar was added (0.26%). Sterilization was done at 121°C for 20 min. Agar NB medium was prepared in Ø 9 cm Petri dishes. The dishes were wrapped with Parafilm to protect microbial contamination.

2.2.2. Rice callus induction

Paddy seeds (*O. sativa* L.) of Aychade were tested in this experiment. The process of callus induction is similar to the prior experiment (*see 1.3.2*). After six weeks, fresh calli from NB agar medium were recovered for 2AP analysis.

2.3. Incubation of rice callus with pyruvic acid

In this experiment 0.5 g of rice calli originating from NB medium with sucrose were immersed in a test tube containing 4 mL sodium phosphate buffer (0.1 M) at pH 8.0 plus 1-pyrroline (4000 ppm) and pyruvic acid (400 ppm) (**Figure 32**). Control was the medium without 1-pyrroline and pyruvic acid. There was also another test with the medium plus 1-pyrroline (4000 ppm). Each treatment was tested in duplicate.

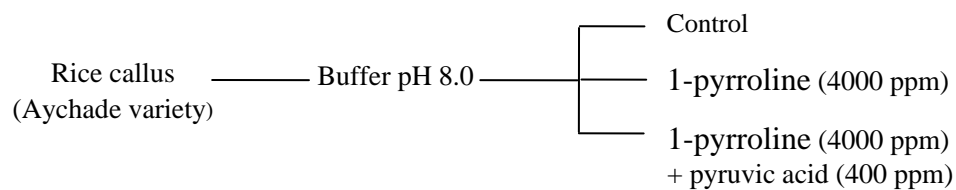


Figure 32. Study of effect of pyruvic acid on the biosynthesis of 2AP in rice calli

2.4. Extraction of 2AP from rice calli

Following incubations rice calli was recovered, ground in liquid nitrogen and 2AP was extracted by SPME technique (*see 1.5.*).

2.5. 2AP analysis

2AP from rice calli was quantified by stable isotope dilution assay as described in *part 1.6.*

For the experiments with C13-labelled compounds, GC-MS in EI, CI as well as PCI-MS/MS mode were used to identify labeled 2AP (*see 2.3.4. in Experiment 2*) by compared with non labeled 2AP.

Part 2

Results and Discussion

1. PUBLICATION No. 3

Some clues on 2-acetyl-1-pyrroline biosynthesis, a major flavouring compound in fragrant rice : key role of 1-pyrroline and source of acetyl group

Submission envisaged beginning 2012

**Janchai POONLAPHDECHA^{a,b}, Pascal GANTET^{c,d}, François-Xavier SAUVAGE^e,
Chantal MENUT^f, Alain MORERE^f, Renaud BOULANGER^b, Ziya GUNATA^{a*}**

^a UMR Qualisud, Université de Montpellier II, place E. Bataillon, 34095 Montpellier Cedex 5, France

^b UMR Qualisud, CIRAD, 73 Rue J. F. Breton, 34398 Montpellier Cedex 5, France

^c Université Montpellier 2, UMR DAP, Bat 15, CC 002, Place Eugène Bataillon, 34095 Montpellier Cedex 5, France

^d University of Science and Technology of Hanoi, Department of Biotechnology and Pharmacology, Cau Giay, Hanoi, Vietnam

^e UMR1083 Sciences pour l'Oenologie, INRA-SupAgro, 2 place Viala, 34060 Montpellier, France

^f IBMM, UMR 5247, Bâtiment E, Faculté de Pharmacie, 15 avenue Charles Flahault, BP 14491, 34093 Montpellier, France

*Corresponding author. Tel. (33) 4 67 14 93 21 - Fax (33) 4 67 14 42 92

e-mail : zgunata@univ-montp2.fr

Part 2



Conclusions

Studies on the biosynthesis of 2AP in rice were performed using rice callus model. Since proline was proposed to be the main amino acid precursor for 2AP formation, firstly we have confirmed that our rice callus was able to increase 2AP synthesis in the presence of proline. Following work focused on the role of 1-pyrroline. Its incubation with fresh intact rice callus both from fragrant rice variety (Aychade) and non fragrant rice variety (Gladio x Fidji-K2) led to a significant increase in 2AP levels. Enzyme-mediated catalysis occurred very probably for the acetylation of 1-pyrroline to generate 2AP. Indeed the formation of 2AP was negligible when rice calli was heated at high temperature prior to incubation with 1-pyrroline. Synthesis of 1-pyrroline was higher at pH 8.0 than pH 5.8 that may be attributed to a better stability of 1-pyrroline at pH 8.0. Furthermore it might be possible that acetyl group transferring enzyme is more active at pH 8.0. Acetyl source seemed not to be limiting factor for the biosynthesis of 2AP. This was supported by two results. Firstly, 2AP could be synthesized by supplementation of a non fragrant rice callus with 1-pyrroline. Secondly when rice callus was supplemented with increasing level of 1-pyrroline (1000 to 7000 ppm) synthesis of 2AP increased almost linearly (560 to 1300 ppb). Overall data highlight that 1-pyrroline is a limiting factor for 2AP synthesis both in fragrant and non fragrant rice varieties.

Incubation of rice callus with pyruvic acid and 1-pyrroline led to an increase in 2AP compared to the control. Pyruvic acid could have been used as acetyl source. The origin of acetyl group was further investigated through generation of rice callus in the presence of C13-labeled putative precursors: [U-13C]6-glucose, [13C]2-sodium acetate and [13C]4-sodium octanoate. With [U-13C]6-glucose 7 possible isotopomers of [13C]-2AP (m/z 111-117) were observed. Hence glycolyse pathway may have generated different intermediates that had been used in the synthesis of 2AP. The presence of isotopomer [13C] 2-2AP and its fragment ion m/z 45 suggested that two carbon-13 labels from glucose were incorporated as acetyl group. Furthermore supplementation of rice callus growth medium with [13C]2-sodium acetate and [13C]4-sodium octanoate gave rise to an isotopomer at m/z 113 at low intensity by EI/MS analysis. CI-MS-MS analysis showed an increase of 2 mass units for two daughter ions (m/z 72, 86) compared to daughter ions from non labelled 2AP (m/z 70, 84). This suggests incorporation of two carbon-13 labels into 1-pyrroline ring through acetyl CoA issued from β -oxidation of aforementioned fatty acids.

General Conclusions

Perspectives

Among several hundred volatiles from fragrant rice, nowadays it has been clearly established that 2-Acetyl-1-pyrroline (2AP) is the key aroma compound. It possesses a very low odor threshold (0.1 ppb in water) and develops a characteristic roasty popcorn-like flavor of cooked rice. It has been suggested that biosynthesis of 2AP in rice is governed not only by genetic factor but also by environmental factors, such as abiotic stresses by the salt and drought. According to literature salt stress may induce synthesis of 2AP through the activation of proline synthesis, a major amino acid precursor of 2AP in rice. However little is known about the relation between proline and 2AP biosynthesis under salt stress. Similarly although biosynthetic pathways of 2AP in rice have been proposed (Bradbury *et al.*, 2008; Huang *et al.*, 2008) the crucial steps, i.e., availability of 1-pyrroline and source of acetyl group have not been demonstrated yet. This thesis aimed to respond to these questions.

The effect of salt treatment during rice plant growth on 2AP synthesis was studied in seedling model and in pots in a greenhouse. Both experiments were performed with Aychade, a fragrant rice variety from Camargue area in France.

Rice seedlings were cultivated in liquid medium for one week under four levels of NaCl solution: 1.7, 17, 85 and 170 mM. Salt solution did not effect strongly growing of rice seedlings. 2AP levels were determined with a newly developed accurate stable isotope dilution assay involving SPME-GC-MS/MS analysis (Maraval *et al.*, 2010). A positive correlation between concentration of salt in the culture medium and 2AP amount in seedling shoots was observed. Salinity treatment increased significantly 2AP level compared to the control whatever the salt concentration in liquid medium. On the contrary proline concentration increased only at the two highest levels of NaCl, 85 mM and 170 mM, respectively. Therefore it appears that proline may not be the limiting factor for 2AP biosynthesis in moderate salt treatment conditions (1.7 to 17 mM NaCl). Nevertheless it may increase 2AP biosynthesis in the presence of high salt solutions (85 to 170 mM NaCl) where significant increase was observed in proline amount. In addition, we observed a decline in the concentration of γ -aminobutyric acid (GABA) at 170 mM. In this condition conversion of γ -aminobutyraldehyde (GABald) into GABA by betain aldehyde dehydrogenase (*BADH2*) may have been reduced (Bradbury *et al.*, 2008) since a quite significant increase in 2AP biosynthesis was observed.

Aychade was also grown in pots filled with the soil in a greenhouse under one level of salt solution (30 mM NaCl). This concentration of salt solution displayed an electrical

conductivity (EC) of $3800 \pm 400 \mu\text{S.cm}^{-1}$ enough to induce salt stress according to the literature. Plants were subjected to salt solution placed in trays at different growth phases (vegetative, reproductive and ripening) and for different durations (from 14 to 28 days). There were four treatments (T1-T4). Reverse osmosis water ($\text{EC}_{\text{water}} < 500 \mu\text{S.cm}^{-1}$) was used as control (T0). In general application of salt solution during rice plant cultivation did not affect significantly plant growth and yield components except for a significant decrease in thousand grain weight (TGW) when salt treatment took place at reproductive phase. Timing and duration of application of a salt solution during rice plant growth resulted in changes in 2AP amount both in the leaves and the grains. The highest concentration of 2AP was observed in the leaves (5717 to 6168 $\mu\text{g.kg}^{-1}$ DW) arising from salt treatment during vegetative (I) phase (beginning tillering (BT) to middle tillering (MT) stage), i.e., treatment T1 and whole vegetative phase (BT to panicle initiation (PI) stage), i.e., treatment T3. Importantly, grains issued from all of the salinity treatments showed significant increase in 2AP compared to the control. In particular the salt application during whole vegetative and reproductive phases yielded the highest concentration of 2AP in the grains (998 and 859 $\mu\text{g.kg}^{-1}$ DW respectively). While salinity treatment during vegetative phase (BT to PI stages) could increase significantly the concentration of 2AP both in the leaves and the grains, salinity during reproductive phase (PI to FLO stage) increased significantly 2AP concentration only in the grains. Proline amount increased significantly only in salt-treated plants during vegetative phase. The level of 2AP also increased in those plants. This data corroborates the results in rice seedlings where proline was found to participate to 2AP biosynthesis (Yoshiashi *et al.*, 2002b). Surprisingly there was no relationship between amount of proline and that of 2AP in the grains although significant increase in 2AP level in the leaves was observed under salinity treatments both in vegetative and reproductive phases. Two possibilities could explain this phenomenon. Either 2AP synthesized in the leaves could have been transported into grains or proline was translocated from the leaves into grains where 2AP synthesis occurred. Furthermore in general the changes in GABA during salt treatment, one of two products in the biosynthesis of 2AP pathway based on *BADH2* activity (Bradbury *et al.*, 2008), could not be related to the synthesis of 2AP.

As conclusion of this part, our results give some clues on the management of salinity conditions during rice cultivation to stimulate biosynthesis of 2AP. Salinity during whole vegetative phase could promote a better synthesis of 2AP in grains without affecting grain yield.

Experimental conditions described here could be used on other fragrant rice in order to understand whether a genotype variability exist for 2AP biosynthesis under salinity. In parallel to elucidate the relation of 2AP with proline, 1-pyrroline and GABA, the expression of betain aldehyde dehydrogenase (*BADH2*), Δ 1-pyrroline-5-carboxylate synthetase (*P5CS*) and Δ 1-pyrroline-5-carboxylate reductase (*P5CR*) under salt stress should be studied. To clarify 2AP and proline translocation between leaves and grains, labeled compounds could be administered to the rice plant at different growth phases.

In the second part of this work, origin of acetyl group in 2AP and role of 1-pyrroline on the biosynthesis of 2AP were studied in the rice callus generated from cv. Aychade Firstly we confirmed that L-proline is able to increase the level of 2AP in rice callus in agreement with the previous study conducted with another fragrant rice cultivar (Yoshihashi *et al.*, 2002b). When rice callus was feeded with 1-pyrroline, the concentration of 2AP increased significantly. This increase was pH dependant and was higher at pH 8.0 than pH 5.8. A more important fraction of 1-pyrroline would have been transformed into its ring-opened form at pH 5.8 that could drop the yield of 2AP synthesis. Heat treatment of rice callus prior to addition of 1-pyrroline resulted in a dramatic decrease in the 2AP biosynthesis suggesting that acetylation of 1-pyrroline could be enzymatic origin. Furthermore a dose-dependent relation was found between the level 1-pyrroline and that of 2AP. The data tends to postulate that acetyl donor is not a limiting factor for the biosynthesis of 2AP in the rice. This assumption was corroborated by results of the experiment with callus from a non-fragrant rice variety Gladio x Fidji-K2. When callus from this variety was supplemented with 1-pyrroline (1000 ppm), 2AP level was enhanced by 290-fold. 2AP level was $580 \mu\text{g.kg}^{-1}$ close to that observed in rice callus from Aychade, fragrant cultivar supplemented with 1-pyrroline. The entire data highlight that 1-pyrroline is a limiting factor for 2AP synthesis in rice.

With regard to acetyl source of 2AP, experiments were conducted with rice callus from cv. Aychade Supplementation of rice callus with pyruvic acid resulted in a significant rise in 2AP amount. Pyruvic acid may have been incorporated to acetyl group through pyruvaldehyde intermediate. Acetyl source was further studied through the supplementation of rice callus growth medium with labeled $[\text{U-}^{13}\text{C}]6\text{-glucose}$, $[\text{C}^{13}]2\text{-sodium acetate}$ and $[\text{C}^{13}]4\text{-sodium octanoate}$. In the experiment with $[\text{U-}^{13}\text{C}]6\text{-glucose}$ 7 possible isotopomers of $[\text{C}^{13}]2\text{AP}$ (m/z 111 - 117) together with several fragment ions (m/z 68 - 73; m/z 83 - 88) were observed. This could explain that carbon-13 atoms were not only incorporated on acetyl group of 2AP but also incorporated into 1-pyrroline ring at different positions. Glycolyse pathway could have

generated different intermediates that had been used in the synthesis of 2AP. The detection of isotopomer [^{13}C]2-2AP (m/z 113) together with a fragment ion m/z 45 shows that two carbon-13 labels from glucose were incorporated as acetyl group. Experiments with [^{13}C]2-sodium acetate and [^{13}C]4-sodium octanoate yielded an isotopomer at m/z 113 by EI/MS analysis but at low intensity. An increase of 2 mass units of daughter ions (m/z 72, 86) compared to non labelled 2AP (m/z 70, 84) were also observed in CI-MS-MS analysis. This tends to suggest the incorporation of two carbon-13 labels into 1-pyrroline ring through acetyl CoA generated from β -oxidation of fatty acids.

To further understand the origin of acetyl group of 2AP, the supplementation of rice callus or seedlings with labeled acetyl CoA may have been undertaken. Most importantly nothing is known about enzymatic acetylation of 1-pyrroline ring. Hence there is a challenge to evidence it and elucidate the catalytic mechanism.

References

- Adams, A., and De Kimpe, N.** (2007). Formation of pyrazines and 2-acetyl-1-pyrroline by *Bacillus cereus*. *Food Chemistry*, 101, 1230–1238.
- Akbar, M., and Ponnampereuma, F.N.** (1982). Saline soil of South and Southeast Asia as potential rice lands. In: IRRI, ed. *Rice Research Strategies for the Future*. IRRI, Manila, the Philippines, (pp. 256-281).
- Akbar, M., Yabuno, T., and Nakao, S.** (1972). Breeding for saline-resistant varieties of rice; Variability for salt tolerance among some rice varieties. *Japanese journal of breeding*, 8, 277-284.
- Aloni, B., and Rosenshtein, G.** (1984). Proline accumulation: A parameter for evaluation of sensitivity of tomato varieties to drought stress? *Physiol. Plant*, 61, 231-235.
- Amirjani, M. R.** (2011). Effect of Salinity Stress on Growth, Sugar Content, Pigments and Enzyme Activity of Rice. *International Journal of Botany*, 7(1), 73-81.
- Apse, M. P., and Blumwald, E.** (2002). Engineering salt tolerance in plants. *Current Opinions in Biotechnology*, 13, 146-150.
- Arai, E., and Itani, T.** (2000). Effects of early harvesting of grains on taste characteristics of cooked rice. *Food Science and Technology Research*, 6, 252-256.
- Ashraf, M., and Foolad, M.R.** (2007). Roles of glycine betaine and proline in improving plant abiotic stress resistance. *Environmental and Experimental Botany*, 59 (2), 206-216.
- Ashraf, M., and Harris, P.J.C.** (2004). Potential biochemical indicators of salinity tolerance in plants. *Plant Science*, 166, 3-16.
- Asch, F., Dingkuhn, M., Dörffling, K. and Miezan, K.** (2000). Leaf K/Na ratio predicts salinity induced yield loss in irrigated rice. *Euphytica*, 113, 109-118.
- Asch, F., and Wopereis, M.C.S.** (2001). Responses of field-grown irrigated rice cultivars to varying levels of floodwater salinity in a semi-arid environment. *Field Crops Res.* 70, 121-137.
- Belardi, R. P., and Pawliszyn, J. B.** (1989). The application of chemically modified fused silica fibers in the extraction of organics from water matrix samples and their rapid transfer to capillary columns. *Water Pollution Research Journal of Canada*, 24 (1), 179-191.
- Belitz, H. D., Grosch, W., and Schieberle, P.** (2004). *Food Chemistry*, 3rd revised Edition. Springer Verlag, Berlin Heidelberg, 2004.
- Bethke, P.C., and Drew, M.C.** (1992). Stomatal and non-stomatal components to inhibition of photosynthesis in leaves of *Capsicum annuum* during progressive exposure to NaCl salinity. *Plant Physiology*, 99, 219-226.
- Bhumbla, D.R., and Abrol, I.P.** (1978). Saline and sodic soils. *Proceedings of the IRRI Symposium on Soils and Rice* (International Rice Research Institute, Manila, Philippines), (pp. 719-738).

- Binzel, M.L., and Reuveni, M.** (1994). Cellular mechanisms of salt tolerance in plant cells. *Horticultural Reviews*, 16, 33-70.
- Blank, I., Devaud, S., Matthey-Doret, W., and Robert, F.** (2003). Formation of Odorants in Maillard Model Systems Based on L-Proline as Affected by pH. *J. Agric. Food Chem*, 51(12), 3643-3650.
- Bohnert, H. J. and Jensen, R. G.** (1996). Strategies for engineering water stress tolerance in plants. *Trends in Biotechnology*, 14, 89-97.
- Bonilla, P. S., Aragon, M. L., and Babiera, V. V.** (2001). Management of Salt-affected soil for rice production. In M. H. Viray (ed). *Rice Technology Bulletin*, 40, 7.
- Borsani, O., Valpuesta, V., and Botella, M.A.** (2001). Evidence for a role of salicylic acid in the oxidative damage generated by NaCl and osmotic stress in *Arabidopsis* seedlings. *Plant Physiol*, 126, 1024-1030.
- Bradbury, L.M.T., Gillies, S.A., Brushett, D.J., Waters, D.L.E., and Henry, R.J.** (2008). Inactivation of an aminoaldehyde dehydrogenase is responsible for fragrance in rice. *Plant. Mol. Biol*, 68, 439-449.
- Bradbury, L., Fitzgerald, T. L., Henry, R. J., Jin, Q. S., and Waters, D.** (2005). The gene for fragrance in rice. *Plant Biotechnol. J*, 3, 363-370.
- Buttery, R. G., Juliano, B. O., and Ling, L. C.** (1983a). Identification of rice aroma compound 2-acetyl-1-pyrroline in Pandan leaves. *Chem. Ind*, 478.
- Buttery, R. G., Ling, L. C. and Juliano, B. O.** (1982). 2-Acetyl-1-pyrroline: an important aroma component of cooked rice. *Chem. Ind*. 23, 958-959.
- Buttery, R.G., Ling, L.C., Juliano, B.O., and Turnbaugh, J.G.** (1983b). Cooked rice aroma and 2-Acetyl-1-pyrroline. *J. Agric. Food chem*, 31, 823-826.
- Buttery, R. G., Ling, L. C., and Mon, T. R.** (1986). Quantitative analysis of 2-acetyl-1-pyrroline in rice. *J. Agric. Food Chem*, 34, 112-114.
- Buttery, R. G., Stern, D. J., and Ling, L. C.** (1994). Studies on Flavor Volatiles of Some Sweet Corn Products. *J. Agric. Food Chem*, 42, 791-795.
- Buttery, R. G., Turnbaugh, J. G., and Ling, L. C.** Contribution of volatiles to rice aroma. *J. J. Agric. Food Chem*, 1988, 36, 1006-1009.
- Champagne, E.T.** (2008). Rice aroma and flavor: a literature review. *Cereal Chem*, 85(4), 445-454.
- Champagne, E.T., Bett-Garber, K.L., Thompson, J., Mutters, R., Grimm, C.C., and McClung, A.M.** (2005). Effects of drain and harvest dates on rice sensory and physicochemical properties. *Cereal Chem*, 82, 369-374.
- Chu, C.C.** (1978) The N6 medium and its applications to anther culture of cereal crops. In Proc Symp on Plant Tissue Cult, May 25-30, Beijing, *Science Press*, China, (pp 43-50).

- Costello, P.J., and Henschke, P.A.** (2002). Mousy Off-Flavor of Wine: Precursors and Biosynthesis of the Causative N-Heterocycles 2-Ethyltetrahydropyridine, 2-Acetyltetrahydropyridine, and 2-Acetyl-1-pyrroline by *Lactobacillus hilgardii* DSM 20176. *J. Agric. Food Chem*, 50, 7079-7087.
- Costello, P.J., Lee, T.H., and Henschke, P.A..** (2001). Ability of lactic acid bacteria to produce Nheterocycles causing mousy off-flavor in wine. *Australian Journal of Grape and Wine Research*, 7(3), 160-167.
- Datta, S. K. D.** (1981). PRINCIPLES AND PRACTICES OF RICE PRODUCTION A Wiley-Interscience publication, John Wiley & Sons, Inc. Printed in Singapore, (pp 640).
- Delauney, A.J., and Verma, D.P.S.** (1993). Proline biosynthesis and osmoregulation in plants, *Plant J.* 4, 215-223.
- Dobermann, A., and Fairhurst, T.H.** (2002). Rice Production (Better Crops International Vol. 16, Special Supplement May 2002. (pp. 7-10)
- Dubey, R.S.** (1994). Protein Synthesis by Plants under Stressful Conditions. In: Handbook of Plant and Crop Stress, Pessaraki, M. (Ed.). Marcel Dekker, New York, (pp. 277-299).
- Efferson, J. N. E.** 1985. Rice quality in world markets. In IRRI ed., Rice Grain Quality and Marketing. IRRI. Manila. 1-13.
- Engel, W., Bahr, W., and Schieberle, P.** (1999). Solvent assisted flavour evaporation-a new and versatile technique for the careful and direct isolation of aroma compounds from complex food matrixes. *Eur. Food Res. Technol*, 209, 237-241.
- FAO** (2001). "Lecture notes on the major soils of the world."
<ftp://ftp.fao.org/agl/agll/docs/wsrr94e.pdf>
- FAO** (2006). World agriculture: Towards 2030/2050. Prospects for food, nutrition, agriculture and major commodity groups. Global Perspective Studies Unit Food and Agriculture Organization of the United Nations, Rome, Italy.
- FAO** (2010). <http://www.fao.org/docrep/012/a1186e/a1186e00.pdf>
(FAO Rice Market Monitor, Trade and Markets Division Food and Agriculture Organization of the United Nations. April 2010 Volume XIII, Issue No. 1).
- Fazzalari, F. A.** (1978). Ed. Compilation of Odor and Taste Threshold Values Data; ASTM Data Series Publication, DS 48A; American Society for Testing and Materials: Philadelphia, PA.
- Ferrero, A., and Nguyen, N.V.,** (2004) Constraints and opportunities for the sustainable development of rice-based production systems in Europe FAO Rice Conference, 12-13 February 2004, FAO, Rome, Italy .
- Fitzgerald, M.A., Hamilton, N.R.S., Calingacion, M.N., Verhoeven, H.A., and Butardo, V.M.** (2008a). Is there a second fragrance gene in rice? *Plant Biot. J*, 6, 416-423.

- Fitzgerald M.A., McCouch, S.,R., and Hall, R.D.** (2009). Not just a grain of rice: the quest for quality. *Trends Plant Sci*, 14, 133-9.
- Fitzgerald, T. L., Waters, D. L. E., and Henry, R. J.** (2008b). The effect of salt on betaine aldehyde dehydrogenase transcript levels and 2-acetyl-l-pyrroline concentration in fragrant and non-fragrant rice (*Oryza sativa*). *Plant Science*, 175, 539-546.
- Flowers, T.J.** (1988). Chloride as a nutrient and as an osmoticum. *Adv Plant Nutr*, 3, 55-78.
- Flowers, TJ, and Flowers SA.** (2005). Why does salinity pose such a difficult problem for plant breeders? *Agricultural Water Management*, 78, 15-24.
- Flowers, T.J., Hajibagheri, M.A., and Clipson, N.J.W.** (1986). Halophytes. *Q. Rev. Biol*, 61, 313-337.
- Flowers., T.J., and Yeo, A.R.** (1981). Variability of sodium chloride resistance within rice (*Oryza sativa* L.) varieties. *New Phytol*, 88, 363-373.
- Food and Agricultural Policy Research Institute, FAPRI, 2009
<http://www.fapri.iastate.edu>
- Foyer, C., Lelandais, M., Galap, C., and Kunert, K.J.** (1991). Effects of elevated cytosolic glutathione reductase activity on the cellular glutathione pool and photosynthesis in leaves under normal and stress conditions. *Plant Physiol*, 97, 863-872.
- Gamborg, O.L., Miller, R.A., Ojima, K.** (1968) Nutrient requirements of suspension cultures of soybean root cells. *Exp Cell Res*, 50, 50151-158.
- Garcia A.B., Engler J.D., Iyer S., Gerats T., Van Montagu M. and Caplan A.B.** (1997). Effects of osmoprotectants upon NaCl stress in rice. *Plant Physiol*, 115, 159-169.
- Gay, F., Hien, P.P., Thu Huong, N.T., Buu, B.C., and Quoc, H.T.** (2006). Investigation on sources of variability in aromatic quality of a famous traditional scented rice variety grown in Mekong Delta. In: Proceedings of the 2nd International Rice Research Congress, New Delhi, India, 9-13 October 2006, p.121.
- Gay, F., Maraval, I., Roques, S., Gunata, Z., Boulanger, R., Audebert, A., and Mestres, C.** (2010). Effect of salinity on yield and 2-acetyl-l-pyrroline content in grains of three aromatic rice cultivars (*Oryza sativa* L.) in Camargue (France). *Field Crops Res*, 117, 154-160.
- Ghoulam. C., Foursy, A., and Fares. K.** (2002). Effects of salt stress on growth, inorganic ions and proline accumulation in relation to osmotic adjustment in five sugar beet cultivars. *Environ. Exp. Bot*, 47 (1), 39-50.
- Giraud, G., Wajid, S., Pirzada, H., and Cesaer, U.M.R.** (2010). Aromatic rice, trade-off between exports and domestic markets, and a global overview. The 28th International Rice Research Conference, 8-12 November 2010, Hanoi, Vietnam. OP13: Policy, Market, and Supply Chain.

- Glazmann, J. C.** (1987). Isozyme and classification of Asian rice varieties. *Theor. Appl. Genet*, 74, 21-30.
- Gorham, J.** (1995). Mechanisms of salt tolerance of halophytes. In: *Halophytes and Biosaline Agriculture*, (Eds. Choukr-Allah R, Malcolm CV. Hamdy A.). Marcel Dekker, Inc. New York Basel, Hong Kong. p. 31-53.
- Grattan, S.R., Zeng, L., Shannon, M.C., and Roberts, S.R.** (2002). Rice is more sensitive to salinity than previously thought. *Calif. Agric*, 56 (6), 189-195.
- Greenway, H., and Munns, R.** (1980). Mechanisms of salt tolerance in non-halophytes. *Ann Rev Plant Physiol*, 31,149-190.
- Grieve, C.M., Lesch, S.M., Maas, E.V., and Francois, L.E.** (1993). Leaf and spikelet primordia initiation in salt-stressed wheat. *Crop Sci*, 22,1286-1294.
- Grimm, C. C., Bergman, C., Delgado, J. T., and Bryant, R.** (2001). Screening for 2-Acetyl-1-pyrroline in the Headspace of Rice Using SPME/GC-MS *J. Agric. Food Chem*, 49, 245-249.
- Grover, A., and Pental, D.** (2003). Breeding objectives and requirements for producing transgenic for the major field crops of India. *Curr Sci*, 84, 310-320.
- Grumet, R., Ialeib, T.G., and Hanson, A.D.** (1985). Genetic control of glycinebetaine level in barley. *Crop Sci*, 25, 618-622.
- Gueta-Dahan, Y., Yaniv, Z., Zilinskas, B.A., and Ben-Hayyim, G.** (1997). Salt and oxidative stress: similar and specific responses and their relation to salt tolerance in Citrus. *Planta*, 203, 460-469.
- Hare, P.D., Cress, W.A., and Van Standen, J.** (1999). Proline synthesis and degradation: the model goes elucidating stress related signal transduction. *J Exp Bot*, 50, 413-34.
- Hasegawa P.M., Bressan R.A., Zhu J.K., and Bohnert, H.J.** (2000). Plant cellular and molecular responses to high salinity. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 51, 463-499.
- Heenan, D.P., Lewin, G. and McCaffery, D.W.** (1988). Salinity, tolerance in rice varieties at different growth stages. *Aust J Exp Agric*. 28, 343-349.
- Hernandez, J.A., and Almansa, M.S.** (2002). Short-term effects of salt stress on antioxidant systems and leaf water relations of pea leaves. *Physiol. Plant.*, 115, 251-257.
- Hien, D.T., Jacobs, M., Angenon, G., Hermans, C., Thu, T.T., Son, L.V., and Roosens, H.** (2003). Proline accumulation and Δ^1 -pyrroline-5-carboxylase synthetase gene properties in three rice cultivars differing in salinity and drought tolerance. *Plant Sci.*, 165 (5), 1059-1068.
- Hofmann, T., Schieberle, P.** (1998). 2-Oxopropanal, hydroxy-2-propanone, and 1-pyrrolines Important intermediates in the generation of the roast-smelling food flavor compounds 2-acetyl-1-pyrroline and 2-acetyltetrahydropyridine. *J. Agric.Food Chem*, 46, 2270-2277.

- Hori, K., Purboyo, R. B. R. A., Akinaga, Y., Okita, T., and Itoh, K.** (1992). Knowledge and preference of aromatic rice by consumers in East and Southeast Asia. *J. Consumer Stud. Home Econ*, 16, 199-206.
- Hori, K., Purboyo, R. B. R. A., Jo, M., Kim, S., Akinaga, Y., Okita, T., and Kang, M.** (1994). Comparison of sensory evaluation of aromatic rice by consumers in East and Southeast Asia. *J. Consumer Stud. Home Econ*, 18, 135-139.
- Houston, D.F.** (1972). Rice hull. Houston, D.F.(ed.). Rice: chemistry and technology. American Association of Cereal Chemists, St. Paul, Minnesota. 301-352.
- Hsieh, R.J.** (1994). Contribution of lipoxygenase pathway to food flavours. Lipids in food flavours. Published by American Chemical Society. Washington, DC. 30-48.
- Hsu, S. Y., Hsu, Y. T. and Kao, C.H.** (2003). The effect of polyethylene glycol on proline accumulation in rice leaves. *Biol. Plant*, 46 (1), 73-78.
- Hu, Y. and U. Schmidhalter.** (1998). Spatial distributions of inorganic ions and sugars contributing to osmotic adjustment in the elongating wheat leaf under saline soil conditions. *Aust J Plant Physiol*, 25, 591-597.
- Huang T.C., Teng C.S., Chang J.L., Chuang H.S., Ho C.T., Wu M.L.** (2008). Biosynthetic mechanism of 2-acetyl-1-pyrroline and its relationship with Δ 1-pyrroline-5-carboxylic acid and methylglyoxal in aromatic rice (*Oryza sativa* L.) callus. *J Agric Food Chem*, 56, 7399-404.
- Iqbal, Z., Ahmad, R., and Hussain, A.** (2000). Studies on Rice Volatiles Using Direct Thermal Analysis Gas Chromatography. *Pakistan Journal of Biological Sciences*, 3 (141), 683-684.
- Ishitani, K. and Fushimi, C.** (1994) Influence of pre-and post-harvest conditions on 2-acetyl-1-pyrroline concentration in aromatic rice. *The Koryo*, 183, 73-80.
- Itani, T., Tamaki, M., Hayata, Y., Fushimi, T. and Hashizume, K.** (2004). Variation in 2-acetyl-1-pyrroline concentration in aromatic rice grains collected in the same region in Japan and factors affecting its concentration. *Plant Prod. Sci*, 7 (2), 178-183.
- Iyengar, E. R. R., and Reddy, M. P.** (1996). Photosynthesis in highly salt tolerant plants. In Pesserkali, M. (Ed.), *Handbook of photosynthesis* New York: Marshal Dekar Baten Rose, (pp. 897-909).
- James, J.J., Tiller, R.L., and Richards, J.H.** (2005). Multiple resources limit plant growth and function in a saline-alkaline desert community. *J. Ecol*, 93, 113-126.
- Jezussek, M., Juliano, B.O., and Schieberle, P.** (2002). Comparison of key aroma compound in cooked brown rice varieties based on aroma extract dilution analyses. *J. Agric. Food. Chem*, 50, 1101-1105.
- Jin, Q. S., D. Waters, G. M. Cordeiro, R. J. Henry, and R. F. Reinke.** (2003). A single nucleotide polymorphism (SNP) marker linked to the fragrance gene in rice (*Oryza sativa* L.). *Plant Sci*, 165, 359-364.

- Kavi Kishor, P.B.** (1988). Effect of salt stress on callus cultures of *Oryza sativa* L. *J. Exp. Bot*, 39, 235-240.
- Kavi Kishor, P.B., Hong, Z., Miao, G.-H., Chein-An, A., Hu, C.A., and Verma, D.P.S.** (1995). Overexpression of D1-pyrroline-5-carboxylate-synthetase increases proline production and confers osmotolerance in transgenic plants, *Plant Physiol*, 108, 1387-1394.
- Kavi Kishor, P.B., Sangarn, S., Amrutha, R.N., Laxrni, P. Sri, Naidu, K.R., Roa, K.R.S.S., Rao, S., Reddy, K.J., Theriappan, P., and Sreenivasulu, N.** (2005). Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: its implications in plant growth and abiotic stress tolerance. *Cwr. Sci*, 88 (3), 424-438.
- Khan, M.H., and Panda, S.K.** (2008). Alterations in root lipid peroxidation and antioxidative responses in two rice cultivars under NaCl-salinity stress. *Acta Physiol. Plant*, 30, 81-89
- Khatun, S., and Flowers. T.J.** (1995). Effects of salinity on seed set in rice. *Plant Cell Environ*, 18, 61-67.
- Khatun, S., Rizzo, C.A., and Flowers, T.J.** (1995). Genotypic variation in the effect of salinity on fertility in rice. *Plant Soil*, 173, 239-250.
- Khush, G. S.** (2000). Taxonomy and origin of rice. In: R. K. Singh, U.S . Singh and G. S. Khush (eds.). *Aromatic Rices*. Oxford & IBH Pub. Co. New Delhi, (pp. 5-13).
- Kim, D.W., Shibato, J., Agrawa, G.K., Fujihara, S., Iwahashi, H., Kim, D.H., Shim, I.-S., and Rakwal, R.** (2007). Gene Transcription in the Leaves of Rice Undergoing Salt-induced Morphological Changes (*Oryza sativa* L.) *Mol. Cells*, 24(1), 45-59.
- Kinnersley, A.M., and Turano, F.J.** (2000). Gama aminobutyric acid (GABA) and plant responses to stress, *Crit. Rev. Plant Sci*, 19, 479-509.
- Kiyosue, T., Yoshiba, Y., Yamaguchi-Shinozaki, K., and Shinozaki, K.** (1996). A nuclear gene encoding mitochondrial proline dehydrogenase, an enzyme involved in proline metabolism, is upregulated by proline but downregulated by dehydration in *Arabidopsis*. *Plant Cell*, 8, 1323-1335.
- Kopittke, P., and Menzies, N.** (2005). Effect of pH on Na induced Ca deficiency. *Plant Soil*, 269, 119-129.
- Kueneman, E.A.** (2006). Improved rice production in a changing environment: from concept to practice. *IRC Newsletter*, 55, 1-20.
- Lafitte, H.R., Ismail, A., and Bennett, J.** (2004). Abiotic stress tolerance in rice for Asia: Progress and the future. In: T. Fischer, N. Turner, J. Angus, L. McIntyre, M. Robertson, A. Borrell, et al. eds. *New Directions for a Diverse Planet*. Proceedings for the 4th International Crop Science Congress. The Regional Institute Ltd.

- Läuchli, A., and Epstein, E.** (1990). Plant responses to saline and sodic conditions. In K.K. Tanji (ed). Agricultural salinity assessment and management. ASCE manuals and reports on engineering practice. ASCE New York, No, 71. (pp. 113-137).
- Levitt, J.** (1980). Responses of plants to environmental stresses. V. II. Physiological ecology. Academic Press, New York, (pp. 365-384).
- Lorieux, M., Petrov, M., Huang, Guiderdoni, E. and Ghesquiere, A.** (1996). Aroma in rice : genetic analysis of quantitative trait. *Theor. Appl. Genet*, 93, 1145-1151.
- Lutts, S., J.M. Kinet and J. Bouharmont.** (1995). Changes in plant response to NaCl during development of rice (*Oryza sativa* L.) varieties differing in salinity resistance. *J. Exp Bot*, 46, 1843-1852.
- Maas, E. V., and Grattan, S. R.** (1999). Crop yields as affected by salinity. In R. W. Skaggs and J. van Schilfgaarde (eds) Agricultural Drainage. Agron. Monograph 38. ASA, CSSA, SSA, Madison, WI, (pp. 55-108).
- Maas, E.V., and Poss, J.A.** (1989). Salt sensitivity of wheat at different growth stages. *Irrig. Sci*, 10, 29-40.
- Maga, J. A.** (1984). Rice Product Volatiles: A Review. *J. Agric. Food Chem*, 32, 904-970.
- Mahatheeranont, S., Keawsa-ard, S., and Dumri, K.** (2001). Quantification of the Rice Aroma Compound, 2-Acetyl-1-pyrroline, in Uncooked Khao Dawk Mali 105 Brown Rice *J. Agric. Food Chem*, 49, 773-779.
- Mansour, M. M. F.** (2003). Nitrogen containing compounds and adaptation of plants to salinity stress. *Biologia Plantarum*, 43, 491-500.
- Maraval, I., Sen, K., Agrebi, A., Menut, C., Morere, A., Boulanger, R., Gay, F., Mestres, C., and Gunata, Z.** (2010). Quantification of 2-acetyl-1-pyrroline in rice by stable isotope dilution assay through headspace solid-phase microextraction coupled to gas chromatography-tandem mass spectrometry. *Anal. Chim. Acta*, 675, 148-155.
- Maraval, I., Mestres, C., Pernin, K., Ribeyre, F., Boulanger, R., Guichard, E., and Gunata, Z.** (2008). Odor-Active Compounds in Cooked Rice Cultivars from Camargue (France) Analyzed by GC-O and GC-MS. *J. Agric. Food Chem*, 56, 5291-5298.
- Misra. N., and Gupta, A.K.** (2005). Effect of salt stress on proline metabolism in two high yielding genotypes green gram. *Plant Sci*, 169 (2), 331-339.
- Misra, A.N., Sahu, S.M., Misra, M., Singh, P., Meera, I., Das, N., Kar, M., and Sahu, P.** (1997). Sodium chloride induced changes in leaf growth, and pigment and protein contents in two rice cultivars, *Biol. Plant*, 39, 257-262.
- Moradi, F., and Ismail, A.M.** (2007). Responses of photosynthesis, chlorophyll fluorescence and ROS-scavenging systems to salt stress during seedling and reproductive stages in rice. *Ann. Bot*, 99, 1161-1173.

- Moradi, F., Ismail, A., Gregorio, G. B., and Egdane, J.** (2003). Salinity tolerance of rice during reproductive development and association with tolerance at the seedling stage. *Indian J Pl Physiol*, 8, 105-116.
- Munns, R.** (2002). Comparative physiology of salt and water stress. *Plant, Cell and Environment*, 25, 239-50.
- Munns, R.** (2005). Genes and salt tolerance: bringing them together. *New Phytol*, 167, 645-663.
- Munns R., James R.A. and A. Läuchli.** (2006). Approaches to increasing the salt tolerance of wheat and other cereals. *J. Exp. Bot*, 57, 1025-1043.
- Murashige, T. and Skoog, F.** (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physio. Plant*, 15, 473-497.
- OECD** (1999). Consensus Document on the Biology of *Oryza sativa* (Rice) No. 14, 1999, Report No. ENV/JM/MONO(99)26, OECD Environmental health and Safety Publications, Paris.
- OECD.** (2004). Consensus Document on Compositional Considerations for New Varieties of Rice (*Oryza Sativa*): Key Food and Feed Nutrients and Anti-Nutrients. Series on the Safety of Novel Foods and Feeds, No.10. ENV/JM/MONO(2004)15.OECD Environment, Health and Safety Publications, Paris.
- Parvaiz, A., and Satyawati S.** (2008). Salt stress and phyto-biochemical responses of plants - a review *Plant Soil Environ*, 54, 89-99.
- Peng, Z., Lu, Q. and Verma, D. P. S.** (1996). Reciprocal regulation of D1-pyrroline-5-carboxylate synthetase and proline dehydrogenase genes control levels during and after osmotic stress in plants. *Mol. Gen. Genet*, 253, 334-341.
- Pingali P, and Hossain M,** editors. (1998). Impact of rice research. Proceedings of the International Conference on the Impact of Rice Research, 3-5 Jun 1996, Bangkok, Thailand. Thailand Development Research Institute, Bangkok, Thailand, and International Rice Research Institute, P.O. Box 933, Manila, Philippines, (pp.428).
- Pinson, S. R. M.** (1994). Inheritance of aroma in six rice cultivars. *Crop Sci*, 34 , 1151-1157.
- Raven, J.A.** (1985). "Regulation of pH and generation of osmolarity in vascular plants: a cost benefit analysis in relation to efficiency of use of energy, nitrogen and water." *New Phytologist*, 101, 25-77.
- Reddy, M. P., Sanish, S., and Iyengar, E. R. R.** (1992). Photosynthetic studies and compartmentation of ions in different tissues of *Salicornia brachiata* Roxb. under saline conditions. *Photosynthetica*, 26, 173-179.
- Ren, Z.H., J.P. Gao, L.G. Li, X.L. Cai, W. Huang, D.Y. Chao, M.Z. Zhu, Z.Y. Wang, S. Luan and H.X. Lin.** (2005). A rice quantitative trait locus for salt tolerance encodes a sodium transporter. *Nature Genet*, 37, 1141-1146.

- Rengasamy, P** (2002), Clay dispersion, In 'Soil physical measurement and interpretation for land evaluation', (Eds: McKenzie, N., Coughlan, K., and Cresswell, H.) CSIRO, Melbourne. (pp. 200-210).
- Richharia, A, Shah, K., and Dubey, R.S.** (1997). Nitrate reductase from rice seedlings: Partial purification, characterization and the effects of in situ and in vitro NaCl salinity. *J. Plant Physiol*, 151,316-322.
- Robacker, D. C., Demilo, A. B., Voaden, D. J.** (1997). Mexican fruit fly attractants: Effects of 1-pyrroline and other amines on attractiveness of a mixture of ammonia, methylamine, and putrescine. *J. Chem. Ecol*, 23, 1263-1280.
- Romanczyk, Jr. L. J., McClelland, C. A., Post, L. S., and Aitken, W. M.** (1995). Formation of 2-acetyl-1-pyrroline by several *Bacillus cereus* strains isolated from cocoa fermentation boxes. *J. Agric. Food Chem*, , 43, 469-475.
- Rondinone, A.** (2007). "For a New Geography of Taste: the Diffusion of Basmati rice in Italy" in Papotti, D., (editors), Geographical Researches on Rice: A comparative Analysis of Rice Districts in the European Union and India, Vercelli, Edizioni Mercurio 2007, (pp. 229-243).
- Rohilla, R., Singh, V.P., Singh, U.S., Singh, R.K., and Khush, G.S.** (2000). Crop husbandry and environmental factors affecting aroma and other quality traits. In: Singh, R.K., Singh, U.S., Khush, G.S. (Eds.), Aromatic Rices. Science Publishers, Enfield, New Hampshire, (pp. 201-216).
- Rychlik, M., and Grosch, W.** (1996). Identification and Quantification of Potent Odorants Formed by Toasting of Wheat Bread. *Lebensm.-Wiss. Technol*, 29, 515-525.
- Sagar, M.A., and Ali, C.A.** (1993). Relationship of Basmati 370 grain quality to soil and environment. *Int. Rice Res. Notes*, 18 (2), 11-12.
- Schieberle, P.** (1991). Primary odorants of popcorn. *J. Agric. Food Chem*, 39, 1141-1144.
- Schieberle, P.** (1996). Odour-active Compounds in Moderately Roasted Sesame. *Food Chem.*, 55, 145-152.
- Serraj R, and Sinclair, TR.** (2002). Osmolyte accumulation: can it really help increase crop yield under drought conditions? *Plant Cell Environ*, 25, 333-41
- Serrano R, and Gaxiola R.** (1994). Microbial models and salt stress tolerance in plants. *CRC Crit Rev Plant Sci*, 13, 121-133.
- Shabala, S.N., Shabala, L., Van Volkenburgh, E. and Newman, I.** (2005). Effect of divalent cations on ion fluxes and leaf photochemistry in salinised barley leaves. *J Exp Bot*, 56, 1369-1378.
- Sharma S.S., and Dietz K.J.** (2006). The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. *J Exp Bot*, 57, 711-26.

- Shi, D., and Wang, D.** (2005). Effects of various salt-alkaline mixed stresses on *Aneurolepidium chinense* (Trin.) Kitag. *Plant Soil*, 271, 15-26.
- Shih, F.F., and Daigle, K.** (1997). Use of Enzymes for the Separation of Protein from Rice Flour. *Cereal Chem*, 74(4), 437-441.
- Shimose, N.** (1995). "Salt and acid sulfate injuries. In: Matsuo, T., Kumazawa, K., Ishii, R., Ishihara, K. and Hirata, H. eds. Science of the rice plant." *Food and Agriculture Policy Research Center, Tokyo*, 2, 983-991.
- Srisedak, T., Wongpornchai, S., and Kitsawatpaiboon, P.** (2006). Rapid Method for Quantitative Analysis of the Aroma Impact Compound, 2-Acetyl-1-pyrroline, in Fragrant Rice Using Automated Headspace Gas Chromatography. *J. Agric. Food Chem*, 54, 8183-8189.
- Struve, C., and Christophersen, C.** (2003) Structural equilibrium and ringchain tautomerism of aqueous solutions of 4-aminobutyraldehyde. *Heterocycles*, 60, 1907-1914.
- Sunthonvit, N.** (2005). Effects of High-Temperature Drying on the Flavor Components in Thai Fragrant Rice. *Drying Technology*, 23(7), 1407 - 1418.
- Suprasanna, P., Ganapathi, T. R., Ramaswamy, N. K., Surendranathan, K. K., and Rao, P. S.** (1998). Aroma synthesis in cell and callus cultures of rice. *Rice Genet/CI Newsletters*, 15, 123-125.
- Taylor, C. B.** (1996) Proline and water deficit: Ups and downs. *Plant Cell* 8, 1221-1224.
- Tester, M. and Davenport, R.** (2003). Na⁺ tolerance and Na⁺ transport in higher plants. *Ann Bot*, 91, 503-527.
- Thomson, W.W., Faraday, C.D., and Cross J.W.** (1988). Salt glands. In: Baker, DA, Hall J.L. (Eds.). *Solute Transport in Plant Cells and Tissues*. Longman Scientific and Technical, Harlow, Essex, England, (pp. 498-537).
- Treichel, S.** (1986) The influence of NaCl on Δ 1-pyrrolinecarboxylate reductase in proline-accumulating cell suspension cultures of *Mesembryanthemum rodiflorum* and other halophytes. *Plant Pysiol*, 67, 173-181.
- Ueda. A. Y. Yamamoto-Yamane and T. Takabe.** (2007). Salt stress enhances proline utilization in the apical region of barley roots. *Biochem. Biophys. Res. Commun*, 335(1), 61-66.
- Vanavichit, A.,** 2007. Molecular Diversity and Evolution of Aromatic Rice in Thailand. International Training-Workshop (2007) The Conservation and Utilization of Tropical/Subtropical Plant Genetic Resources, 131-134.
- Vanavichit A, Yoshihashi T, Wanchana S, Areekit S, Saengsraku D, Kamolsukyonyong, W, et al.** (2005). Cloning of Os2AP, the aromatic gene controlling the biosynthetic switch of 2-acetyl-1-pyrroline and gamma aminobutyric acid (GABA) in rice. 5th International Rice Genetics Symposium. Philippines: IRRI, 2005. Nov 19-23, (pp.44).

- Verbruggen, N., Hua, X.J., May, M., and Van Montagu, M.** (1996). Environmental and developmental signal modulate proline homeostasis: evidence for a negative transcriptional regulator. *Proc. Natl. Acad. Sci, U.S.A.* 93, 8787-91.
- Voetberg G.S., and Sharp R.E.** (1991). Growth of the maize primary root in low water potentials. III. Roles of increased proline depositions in osmotic adjustment. *Plant Physiol*, 96, 125-30.
- Wang. Z.Q., Yuan, Y.Z. , Ou,. J.Q., Lin, Q.H., and Zhang, C.F.** (2007). Glutamine synthetase and glutamate dehydrogenase contribute differentially to proline accumulation in leaves of wheat (*Triticum aestivum*) seedlings exposed to different salinity. *J. Plant Physiol*, 164 (6), 695-701.
- Weber, D.J. Rohilla, R. and Singh, U.S.** (2000). Chemistry and biochemistry of aroma. In: Singh, R.K., Singh, U.S., Khush, G.D (Eds.). Aromatic rices. Oxford and IBH Publishing Co. Pvt. Ltd., New Dehli, India, (pp. 29-46).
- White, I., Melville, M. and Sammut, J.** (1996). "Possible impacts of saline water intrusion floodgates in Vietnam's Lower Mekong Delta." Seminar on environment and development in Vietnam. AusAID, International Seminar Support Scheme.
http://coombs.anu.edu.au/~vern/env_dev/papers/pap07.html (date 20/06/2006)
- Widjaja, R., Craske, J. D., and Wootton, M.** (1996). Comparative studies on volatile components of non-fragrant and fragrant rices. *J. Sci, Food Agric*, 70, 151-161.
- Wilkie K., and Wootton M.** (2004). Flavour qualities of new Australian fragrant rice cultivars; 2004. RIRDC Publication No 04/ 160 RIRDC Project No UNS-12A
- Wongpornchai, S., Sriseadka, T., and Choonvisase, S.** (2003) Identification and Quantitation of the Rice Aroma Compound, 2-Acetyl-1-pyrroline, in Bread Flowers (*Vallaris glabra* Ktze). *J. Agric. Food Chem*, 51, 457-462.
- Wu, R., Kemmercr, E., and McElroy, D.** (1990). Gene manipulation in plant improvement. 11, 251-263.
- Wu, C.J., Cheng, Z. Q., Huang, X. Q., Yin, S. H., Cao, K. M., and Sun, C. R.** (2004). Genetic diversity among and within populations of *Oryza granulata* from Yunnan of China revealed by RAPD and ISSR markers: implications for conservation of the endangered species. *Plant Science*, 167, 35-42.
- Yamamoto, A., Bhuiyan, M.N.H., Waditee. R., Tanaka. Y., Esaka. M., Oba. K., Jagendorf, A,T, and Takabe, T.** (2005). Suppressed expression of the apoplastic ascorbate oxidase gene increases salt tolerance in tobacco and *Arabidopsis* plants. *J. Exp. Bot.*, 56, 1785-1796.
- Yajima, I., Yanai, T., Nakamura, M., Sakakibara, H., and Hayashi K.** (1979). Volatile flavor components of cooked Kaorimai (scented rice *O. sativa japonica*). *Agric. Biol. Chem*, 43, 2425-2429.
- Yancey P.H., Clark, M.E., Hand, S.C., Bowlus, R.D., and Somero G.N.** (1982). Living with water stress: evolution of osmolyte systems. *Science*, 217, 1214-1222.

- Yang, C., Shi, D., and Wang, D.** (2008). Comparative effects of salt and alkali stresses on growth, osmotic adjustment and ionic balance of an alkali-resistant halophyte *Suaeda glauca* (Bge.). *Plant Growth Regul*, 56, 179-190.
- Yeo, A.R., and Flowers, T.J.** (1986). Salinity resistance in rice (*Oryza sativa* L.) and a pyramiding approach to breeding varieties for saline soils. In: Effect of drought on plant growth. Salts in soils. N. C. Turner and J. B. Passioura (Eds.). CSIRO. Melbourne. Australia, (pp. 161-173).
- Yeo, A.R., Yeo, M.E., Flowers, S.A., and Flowers, T.J.** (1990). Screening of rice (*Oryza sativa* L.) genotypes for physiological characters contributing to salinity resistance, and their relationship to overall performance. *Theor Appl Genet*, 79, 377-384.
- Yokoi S., Quintero F.J., Cubero B., Ruiz M.T., Bressan R.A., Hasegawa P.M., and Pardo J.M.** (2002). Differential expression and function of *Arabidopsis thaliana* NHX Na⁺/H⁺ antiporters in the salt stress response. *Plant J*, 30, 529-539.
- Yoshida, Y., Kiyosue, T., Nakashima, K., Yamaguchi-Shinozaki, K.Y. and Shinozaki, K.** (1997). Regulation of levels of proline as an osmolyte in plants under water stress. *Plant Cell Physiol*, 38(10), 1095-1102.
- Yoshihashi, T.** (2002a). Quantitative analysis on 2-acetyl-1-pyrroline of an aromatic rice by stable isotope dilution method and model studies on its formation during cooking. *J. Food Sci*, 67, 619-622.
- Yoshihashi, T., Kabaki, N., Nguyen, T.T.H., and Inatomi, H.** (2004a). Formation of flavor compound in aromatic rice and its fluctuations with drought stress. In: *Research Highlights JIRCAS 2002-2003*, pp. 32-33.
- Yoshihashi, T., Nguyen, T.T.H., and Inatomi, H.** (2002b). Precursors of 2-acetyl-1-pyrroline, a potent flavor compound of an aromatic rice variety. *J. Agric. Food Chem*, 50, 2001-2004.
- Yoshihashi, T., Nguyen, T.T.H., and Kabaki, N.** (2004b). Area dependency of 2-acetyl-1-pyrroline content in an aromatic rice variety. Khao Dawk Mali 105. *JARQ* 38 (2), 105-109.
- Zeng, L., and Shannon, M.C.** (2000). Salinity effects on seedling growth and yield components of rice. *Crop Sci*, 40, 996-1003.
- Zeng, L., Shannon, M.C. and Lesch, S.M.** (2001). Timing of salinity stress affects rice growth and yield components. *Agric. Water Manag*, 48, 191-206.
- Zhang C-S, Lu, Q., and Verma, D.P.S.** (1995) Removal of feedback inhibition of D1-pyrroline-5-carboxylate synthetase, the bifunctional enzyme catalyzing the first two steps of proline biosynthesis in plants. *J Biol Chem*, 270, 20491-6.
- Zhifang G., and Loescher W.H.** (2003). Expression of a celery mannose 6-phosphate reductase in *Arabidopsis thaliana* enhances salt tolerance and induces biosynthesis of both mannitol and a glucosyl-mannitol dimer. *Plant Cell Environ*, 26, 275-283.

Zhu, J.K. (2001). Plant salt tolerance. *Trends in Plant Sciences*, 6, 66-71.

Zhu, J.K. (2003). Regulation of ion homeostasis under salt stress. *Current Opinion in Plant Biology*, 6, 441-445.

**Biosynthesis of 2-acetyl-1-pyrroline, a potent flavour compound in rice :
effect of salt stress during plant growth and some clues on the biosynthetic pathway**

Abstract : The effect of salinity during rice plant growth on the biosynthesis of 2-acetyl-1-pyrroline (2AP), characteristic flavour compound of fragrant rice cultivars was studied. Additionally role of 1-pyrroline in 2AP biosynthesis together with source of acetyl group were investigated. Aychade, a fragrant rice was cultivated either in a liquid medium adapted for seedlings or in pots in a greenhouse. A significant increase in 2AP levels in the leaves was observed whatever the concentration of NaCl (1.7 to 170 mM NaCl) in the culture medium. A positive correlation was found between the level of salt and that of 2AP. There was a relationship between the concentration of proline, a major amino precursor of 2AP and 2AP level at highest concentrations of NaCl. Conversely the changes in γ -aminobutyric acid (GABA), a competitive metabolite in 2AP biosynthesis were not correlated with those of 2AP. In greenhouse experiments, Aychade cultivar was grown under one level of salt solution, 30 mM that corresponds to EC of $3800 \pm 400 \mu\text{S.cm}^{-1}$, value considered sufficient to induce salt stress in rice. Timing and duration of application of salt solution varied according to the growth stages. At vegetative phase a positive relation was observed between salt treatment and 2AP as well as proline levels in the leaves. Interestingly grains from all salt treated plants contained significantly higher levels of 2AP than those from control. The highest synthesis occurred when plants were subjected to salt treatment during whole vegetative and reproductive phases. However in the latter case crop yield decreased significantly. Through supplementation of rice callus with 1-pyrroline it was demonstrated that this cyclic imine was limiting factor for the 2AP biosynthesis not only in a fragrant variety but also in a non fragrant variety. Experiments with ^{13}C labelled glucose and fatty acids showed that glucose and fatty acids could be precursors of acetyl group of 2AP.

Key words: rice, fragrant and non fragrant varieties, 2-acetyl-1-pyrroline, biosynthesis, salt, 1-pyrroline, acetyl group

**Biosynthèse de la 2-acetyl-1-pyrroline, composé d'arôme-clé dans le riz :
effet du stress salin pendant le développement de la plante et contribution à la voie biosynthétique**

Résumé : Effet de la salinité pendant la croissance de plants de riz sur la biosynthèse de la 2-acetyl-1-pyrroline (2AP), composé aromatique clé des variétés de riz aromatique, le rôle de la 1-pyrroline dans la biosynthèse de la 2AP ainsi que l'origine du groupe acétyle ont été étudiés. La variété aromatique de Camargue (France), Aychade, a été cultivée soit dans un milieu liquide soit dans des pots en serre. Dans le milieu de culture liquide, une augmentation significative de la teneur en 2AP dans les feuilles a été observée quelle que soit la concentration en NaCl (de 1,7 à 170 mM). Une corrélation positive a été mise en évidence entre la teneur en sel et en 2AP. A de plus fortes concentrations en NaCl, il a également pu être montré qu'il y avait une relation entre la concentration en proline, un précurseur aminé majeur de la 2AP, et la teneur en 2AP. Inversement, les changements en acide γ -aminobutyrique (GABA), un des métabolites compétitif de la biosynthèse de la 2AP, n'ont pas été corrélés avec ceux de la 2AP. Dans les expérimentations menées en serre, la variété Aychade a été cultivée dans une solution saline à 30 mM, correspondant à une conductivité électrique de $3800 \pm 400 \mu\text{S.cm}^{-1}$, valeur considérée comme suffisante pour induire un stress salin sur le plant de riz. Cette solution saline a été appliquée à des dates et pour des durées différentes en fonction du cycle de développement de la plante. A la phase végétative, une relation positive a été observée dans les feuilles entre le traitement salin et la teneur en 2AP ainsi qu'en proline. Il est intéressant de noter que tous les grains provenant des plants traités avec la solution saline contenaient des teneurs nettement plus élevées en 2AP par rapport au contrôle. La synthèse de la 2AP la plus importante s'est produite lorsque les plants ont été soumis à un traitement salin pendant l'ensemble des phases végétative et reproductive. Cependant, dans le dernier cas, le rendement en grain était beaucoup plus faible. En supplémentant des cals de riz avec de la 1-pyrroline, il a été démontré que cette imine cyclique était un facteur limitant pour la biosynthèse de la 2AP non seulement dans la variété aromatique, mais également dans la non aromatique. Les essais conduits avec le glucose et les acides gras marqués ^{13}C ont montré que ces molécules pourraient être précurseurs du groupe acétyle de la molécule de 2AP.

Mots clés : riz, variétés aromatique et non aromatique, biosynthèse, sel, 2-acetyl-1-pyrroline, 1-pyrroline, groupe acétyle

Discipline: Science des Aliments

Intitulé et adresse du laboratoire : UMR Qualisud, Université de Montpellier II, place E. Bataillon, 34095 Montpellier Cedex ; UMR Qualisud, CIRAD, 73 Rue J. F. Breton, 34398 Montpellier Cedex 5, France